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(54) Title: LINEAR AND HAIRPIN OLIGONUCLEOTIDE ANALOGUES COMPRISING INTERCALATOR PSEUDONUCLEOTIDES

(57) Abstract: The present invention relates to hairpin oligonucleotides or oligonucleotide analogues comprising at least one intercalator pseudonucleotide. Intercalator pseudonucleotides are capable of being incorporated into the phosphate backbone of a nucleic acid and comprises an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid. The invention further relates to methods of detecting hybridisation between (parts of) said hairpin oligonucleotides or oligonucleotide analogues and a template. In addition, methods for detecting template directed extension of an oligonucleotide analogue comprising at least one intercalator pseudonucleotides are described. In preferred embodiments of the invention the oligonucleotide analogue is a hairpin.



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## Linear and hairpin oligonucleotide analogues comprising intercalator pseudonucleotides

### Field of the invention

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The present invention relates to the field of pseudonucleotides comprising intercalators. In one embodiment the invention relates to the field of linear and hairpin oligonucleotide analogues comprising said intercalator pseudonucleotides. In particular the invention relates to such oligonucleotide analogues for use in extension and amplification of nucleic acids and nucleic acid analogues.

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Furthermore, the invention relates to the field of detecting hybridisation of said oligonucleotide analogues as well as to incorporation of said oligonucleotide analogues into nucleic acids or nucleic acid analogues and to detection of said intercalator pseudonucleotides in extension products.

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### Background of invention

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Nucleic acids, such as DNA, RNA as well as a number of nucleic acid analogues such as PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA,  $\alpha$ -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA,  $\beta$ -D-Ribopyranosyl-NA,  $\alpha$ -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA,  $\alpha$ -L-RNA,  $\alpha$ -D-RNA,  $\beta$ -D-RNA and others are capable of specifically hybridizing to their complementary strands. This specific recognition has for example been used to detect the presence of specific nucleic acid sequences and to prime extension of specific oligonucleotides or oligonucleotide analogues in a template dependent manner.

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Detection of the presence and amount of specific nucleic acid sequences is used extensively in particularly RNA expression studies. RT-PCR based techniques have become widespread and are now considered an indispensable method for assaying

gene expression. The well-known previous generation detection formats based on end-point quantification of gel-separated e.g. ethidium bromide stained PCR-product or radioactively labeled PCR-product is now often being replaced by fluorescence based detection with new fluorescent molecules. This kind of detection is typically carried out during the PCR process (often in real-time), saving time and providing data of higher quality than using older methods. Several fluorescence based detection systems for specific nucleic acid sequences are available and all are based on one or more primers/probes detecting the presence of the specific nucleic acid sequence.

PCR remains an indispensable tool when validated gene expression studies need to be performed, but the multiplexing and throughput capabilities of PCR still limit its uses in some applications. For genome wide assessment of gene expression, DNA micro array approaches are therefore preferred.

Both PCR and micro array methods however have shortcomings and to overcome these shortcomings, synthetic or modified nucleic acids with new improved hybridization properties have been used.

Several different synthetic nucleic acids exist. Certain synthetic nucleic acids have an increased affinity for nucleic acids in general. High affinity towards target nucleic acids may greatly facilitate hybridization based assays and high affinity may also be useful for a number of other reactions involving nucleic acids for example amplification reactions.

Furthermore, most nucleic acids as well as most synthetic nucleic acid analogues do not discriminate rigidly between different kinds of nucleic acid, i.e. they bind roughly equally well to complementary DNA and complementary RNA.

Pyrene is an excimer-forming molecule, which has been incorporated into oligodeoxynucleotides (ODNs) by several groups. Ebata et al. incorporated a pyrene-modified nucleotide in the 5' end of one ODN and a pyrene-modified nucleotide into the 3' end of another. By hybridizing to a target sequence in a way that the pyrene moieties from the two strands come into close proximity of each other, an excimer band at 490 nm was generated. Paris et al. published a similar system where they

also explored the utility of the system to detect mismatches, but the ability of the system to differentiate between a fully complementary sequence (wt) and a single point mutant (mut) is due to the ability of one of the probes to hybridize in the first case but not in the latter. This means that the phenomena is temperature controlled and limits the length of the probe and hence the selectivity and sets high requirements to the temperature control.

In the prior art synthetic nucleotide like molecules comprising intercalators have been described:

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**US 5,446,578** describes synthetic nucleotide like molecules comprising fluorescent molecules, which show a change in spectra with concentration, for example pyrene. In particular, the document describes nucleic acids derivatised with such fluorescent molecule on the phosphate of a nucleic acid backbone or nucleic acids comprising an acyclic backbone monomer unit consisting of 5 atoms between two phosphates of the nucleic acid backbone, coupled to such a fluorescent molecule. The document states that the fluorescent molecules should be positioned at the exterior of a nucleic acid helix so that they are not capable of intercalating with nucleobases of a nucleic acid. Furthermore, it is explained that the fluorescence of the fluorescent molecule increases upon hybridization and that a cationic surfactant must be present to achieve this effect.

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**Yamana et al., 1999**, describes an oligonucleotide containing a 2'-O-(1-pyrenylmethyl)uridine at the center position. Said oligonucleotide has higher affinity for DNA and lower affinity for RNA compared to an unmodified oligonucleotide. Upon hybridization monomer and excimer fluorescence is enhanced.

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**Yamana et al., 1997**, describes a phosphoramidite coupled to pyrene, which may be incorporated into a nucleic acid at any desired position. In particular said phosphoramidite may be incorporated into a nucleic acid, as an acyclic backbone monomer consisting of 5 atoms between two phosphates of the nucleic acid backbone. Upon hybridization, excimer fluorescence is greatly enhanced and nucleic acids into which said phosphoramidites have been incorporated retain normal binding affinity for DNA.

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- Korshun et al., 1999**, describes a phosphoramidite coupled to a pyrene, which may be incorporated into a nucleic acid at any desired position. In particular said phosphoramidite may be incorporated into a nucleic acid, as an acyclic backbone monomer consisting of 5 atoms between two phosphates of the nucleic acid backbone. Furthermore the document describes oligonucleotides into which said phosphoramidites have been incorporated and it is described that the oligonucleotides have higher affinity for DNA, than an unmodified oligonucleotide. It is mentioned that close coplanar mutual approach of two pyrene residues located in the neighboring positions of a modified oligonucleotide chain is strongly inhibited because of the small length of the linker. Excimer fluorescence increases upon hybridization, however oligonucleotides comprising 5 such pyrene pseudonucleotides at the end exhibit high excimer fluorescence when unhybridised as well.
- US 5,414,077** describes pseudonucleotides, which may comprise an intercalator such as an acridine or anthraquinone. The pseudonucleotide comprises an achiral or a single enantiomer organic backbone, such as diethanolamine. The pseudonucleotides may be incorporated at any desired position within an oligonucleotide. Such oligonucleotides in general have higher affinity for complementary nucleotides, in particular when the pseudonucleotides are inserted at the end. The document does not describe fluorescence data.
- US 6,031,091** describes pseudonucleotides which may be incorporated at any position in an oligonucleotide. In particular the document describes acyclic phosphor containing backbones and it is mentioned that the pseudonucleotides may comprise an intercalator. Specific pseudonucleotides described in the document comprise very long linkers connecting polyaromates to the nucleic acid backbone.
- EP 0 916 737 A2** describes polynucleotides derivatised with for example intercalating compounds. The intercalating compounds should preferably be positioned with approx. 10 nucleotides in between. The polynucleotide may be derivatised on the phosphate, the sugar or the nucleobase moiety. In particular, they may be derivatised on the nucleobase by a 7 or a 11 atoms long linker coupled to a polyaromate in

a manner that does not interfere with Watson-Crick base pairing. It is stated that fluorescence intensity is enhanced by intercalation.

5       **Strässler et al., 1999**, describes pseudonucleosides comprising a fluorescent molecule for example pyrene instead of a nucleobase.

10       **WO 97/43298** describes nucleoside analogues comprising a polyaromatic hydrocarbon for example pyrene attached to the 1' position of ribose or deoxyribose as well as phosphoramidite derivatives of said polyaromatic hydrocarbons.

15       **US 5,175,273** describes nucleotide derivatives comprising nucleobases fused to planar polycyclic aromatic compounds. Oligonucleotide comprising said nucleotide derivatives have increased affinity for DNA and fluorescence is decreased by hybridization.

20       **US 6,153,737** describes nucleotide derivatised on the 2' position with pyrene or another intercalator.

25       **Ebata et al., 1995** describes incorporation of a pyrene-modified nucleotide in the 5' end of a DNA oligonucleotide and a pyrene-modified nucleotide into the 3' end of another. By hybridizing to a target sequence in a way that the pyrene moieties from the two strands come into close proximity of each other, an excimer band at 490 nm was generated.

30       **Paris et al., 1998** described a system similar to the one disclosed by Ebata et al. wherein the system may be utilized to detect mismatches. However the ability of the system to differentiate between a fully complementary sequence (wt) and a single point mutant (mut) is due to the ability of one of the probes to hybridize in the first case but not in the latter. This means that the phenomena is temperature controlled and limits the length of the probe and hence the selectivity and sets high requirements to the temperature control.

In the prior art nucleotides and nucleotides analogues capable of forming hairpin structures have been described:

Hairpin-forming oligonucleotide hybridization probes with interactive label pairs, particularly fluorescent label pairs and fluorescence-quencher label pairs, are known. Tyagi et al., PCT application No. WO95/13399; Tyagi et al., PCT application No. WO97/39008; Tyagi and Kramer (1996) Nature Biotechnology 14:303. These probes, labeled with a fluorophore and a quencher, are usually "dark", that is, have relatively little or no fluorescence, when free in solution but fluoresce when hybridized to their nucleic acid targets. They are constructed with a variety of fluorophores and are utilized in both end-point and real-time homogeneous assays, including multiplex assays. Tyagi et al. (1998) Nature Biotechnology 16:49; Kostrikis et al. (1998) Science 279:1228; Piatek et al. (1998) Nature Biotechnology 16:359. Hairpin-containing primers similarly labeled with a fluorophore and a quencher are also known. Nazarenko et al. (1997) Nucleic Acids Research 25:2516.

## Summary of the invention

This application claims benefit under § 119(e) to U.S. provisional patent application Serial No. 60/365,545 filed 20 March 2002, which is hereby incorporated by reference in its entirety.

All patent and non-patent references cited in the application, or in the present application, are also hereby incorporated by reference in their entirety.

End point detection and real-time detection of oligonucleotide or oligonucleotide analogue template directed extension is useful for a large number of different applications. However, there has been a shortage of easy-to-handle, readily available, specific, and cost-effective detection methods with fast kinetics that permit real quantitative and real-time detection as well as a high degree of multiplexing.

Surprisingly, the inventors have discovered, that the insertion of intercalators into oligonucleotides or oligonucleotide analogues results in spectral and thermodynamic properties, which allow for specific hybridization, leveling of melting temperature in different but simultaneous hybridization events and exonuclease stability; which may

be followed by detection of template directed extension of oligonucleotide analogues.

Accordingly, it is a first object of the present invention to provide oligonucleotides or  
5 oligonucleotide analogues comprising at least one intercalator pseudonucleotide of the general structure



10 wherein

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

15 Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

Y is a linker moiety linking said backbone monomer unit and said intercalator;  
and

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and wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides

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In particular, it is an object of the present invention to provide such oligonucleotides or oligonucleotide analogues wherein said second sequence is capable of hybridizing to a homologously complementary target sequence comprised within a nucleic acid or nucleic acid analogue and is capable of priming a template directed  
30 extension reaction; and wherein said first sequence will not hybridise with said second sequence. It is also an object of the present invention to provide hairpin oligonucleotide analogues comprising at least one intercalator pseudonucleotide of the general structure

35



wherein

5 X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

10 Y is a linker moiety linking said backbone monomer unit and said intercalator; and

and wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides  
15 and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein said first sequence is capable of hybridising to said second sequence.

In particular, it is an object of the present invention to provide such hairpin  
20 oligonucleotide analogues, wherein said first sequence and said second sequence are separated by a third sequence consisting of p nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein said first sequence is capable of hybridizing to said second sequence; and wherein the second and third  
25 sequence is capable of hybridizing to a homologously complementary target sequence comprised within a nucleic acid or nucleic acid analogue and is capable of priming a template directed extension reaction.

It is also an object of the present invention to provide methods for detecting template  
30 directed extension of an oligonucleotide analogue comprising intercalator pseudonucleotides

- a) providing the template(s); and
- b) providing at least one oligonucleotide analogue comprising intercalator pseudonucleotide(s); and

- c) incubating said template(s) and said oligonucleotide analogue under conditions allowing for hybridization; and
- d) providing nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides; and
- 5 e) extending the oligonucleotide analogue in a template directed manner; and
- f) detecting said template directed extension.

Furthermore, it is an object of the present invention to provide methods of detecting hybridisation between a hairpin oligonucleotide analogue comprising an intercalator pseudonucleotide as described herein above and a template comprising a nucleic acid sequence capable of hybridising to the second sequence of said hairpin oligonucleotide analogue comprising the steps of

- a) providing the template; and
- 15 b) providing an oligonucleotide or oligonucleotide analogue according to the present invention; and
- c) incubating the template and the oligonucleotide or oligonucleotide analogue under conditions allowing for hybridisation; and
- d) detecting hybridisation.

20

### Legends to figures

**Figure 1** illustrates the synthesis of an intercalator pseudonucleotide, a phosphoramidite as depicted in 5.

**Figure 2** illustrates a structural calculation of the self-complementary DNA duplex with the sequence 5'-XCGCGCG-3' done in "MacroModel", X = the pyrene module. The pyrene moiety is co-axial stacked with the underlying base pair.

30

**Figure 3** illustrates a slice of the calculated structure of the duplex 5'-AGCTTGCCTTGAG-3' + 5'-CTCAAGXCAACCT-3', X = 5. The pyrene makes co-

axial stacking with both the upper and lower neighboring nucleobases of the opposite strand.

5     **Figure 4** illustrates the calculated structure of a 12/13-mer duplex with the sequence 5'-AGCTTGCTTGAG-3' + 5'-CTCAAGXCAACCT-3', X = 5 (figure 1). The pyrene moiety is able to interact with both the upper and lower neighboring nucleobases of the opposite strand. The distance between the nucleobases and the pyrene moiety is shown to the right.

10     **Figure 5** illustrates fluorescent measurements of a 13-mer, mono pyrene inserted ssDNA (★); its duplex with complementary, 12-mer RNA ( ) and its duplex with complementary, 12-mer DNA (◆). The sequences are the same as those shown in Table 3.

15     **Figure 6** illustrates fluorescent measurements of a 14-mer ssDNA with two pyrene insertions separated by one nucleotide (★); its duplex with complementary, 12-mer RNA ( ) and its duplex with complementary, 12-mer DNA (◆). The sequences are the same as those shown in Table 3.

20     **Figure 7** illustrates a procedure to prepare a sample for RT-PCR

**Figure 8** illustrates a procedure to prepare a sample for RT-PCR

**Figure 9** illustrates a procedure to prepare a sample for RT-PCR

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**Figure 10** illustrates a procedure to prepare sequence specific DNA

**Figure 11** illustrates a procedure to prepare a sequence specific DNA

30     **Figure 12** illustrates a method to detect sequence specific DNA using a chip

**Figure 13** illustrates different kinds of oligonucleotides that may be useful as probes on a chip

35     **Figure 14** illustrates PCR quantification.

Figure 15 illustrates transcription blockage using a pair of oligonucleotides according to the invention indicated as A and B, respectively.

5     **Figure 16:** Nuclease resistance of two oligonucleotides whereof one comprises intercalating pseudonucleotides (INA oligo) and the duplex of said two oligonucleotides.

10     **Figure 17:** Secondary structure of the hairpin forming probe I. In this conformation the monomer and excimer fluorescence is quenched.

15     **Figure 18:** Secondary structure of probe I when hybridised to a target sequence. When hybridized to a target sequence, the excimer complex is free to be formed and hence excimer fluorescence can be observed. The monomer fluorescence is also increased.

**Figure 19** SYBR green II stained INA oligos, visualized on an ArrayWorx scanner.

20     **Figure 20:** illustrates a test of oligo binding on Asper SAL slides.

**Figure 21:** Exciplex fluorescence between molecules X and Y when placed as next-nearest neighbours (Sequence I)

25     **Figure 22:** Exciplex fluorescence between molecules X and Y when placed as neighbours (Sequence II)

**Figure 23:** Exciplex fluorescence between molecules Y and Z when placed as neighbours

30     **Figure 24:** illustrates EtBr staining

**Figure 25:** Sequence of the employed double-stranded target oligo, the attacking IOs and the complimentary pairing IOs. Y denote intercalating units.

35     **Figure 26:** IOs spontaneously bind target DNA.



Reactions were carried out in 20 µl volumes containing 126 nM IOs with or without 20 nM target DNA, for 1 h at 37 °C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

5 **Figure 27: IO-DNA complex formation requires sequence complementarity.**

Reactions were carried out in 15 µl volumes containing the indicated concentrations of IOs with or without 20nM target DNA (single or double stranded), for 2 h at 37 °C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

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**Figure 28: IO pairing in spontaneous target binding.**

Reactions were carried out in 15 µl volumes containing 20nM target DNA and the indicated concentrations of pre-annealed P32-labelled IOs for 2 h at 37°C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

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**Figure 29: Pairing does not affect the efficiency of spontaneous binding.**

Reactions were carried out in 15 µl volumes containing 20nM target DNA and increasing amounts of IOs (40-80-160 nM) as indicated for 4 h at 37 °C. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging. Band intensities are relative numbers representing intensities of the band areas.

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**Figure 30: IO-DNA complex formation in nuclear extracts**

Reactions were carried out in 15 µl volumes containing pre-annealed 180 nM IOs and 20 nM target DNA where indicated, nuclear extracts (NE) were added to the reactions as indicated. Reactions were incubated at 37°C for 10 min, and then another 60 min upon addition of 1.125 µl 10% SDS and 37.5 µg Proteinase K. Binding was assayed by electrophoresis in a 7 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging.

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**Figure 31: Nuclear factors favour IO-DNA complex formation by IO pairs**

Reactions were carried out in 15 µl volumes containing 180 nM IOs and 20 nM target DNA. 10 µg HeLa nuclear extract were added to the reactions. Reactions were

incubated at 37°C for 10 min, and then another 60 min upon addition of 1.125  $\mu$ l 10% SDS and 37.5  $\mu$ g Proteinase K. Binding was assayed by electrophoresis in a 10 % polyacrylamide 1/2xTBE gel and visualized by phosphorimaging

5 **Figure 32:** Chemical structures of LNA and INA **P** nucleotide monomers. **B** = nucleobase.

**Figure 33:** Melting temperature data of INAs with different insertion patterns when hybridised to the complementary structure and LNA targets. **P** = INA monomer **P**.  $T^L$  and  $^{Me}C^L$  are locked nucleotides of thymine and 5-methylcytosine, respectively.

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**Figure 34:** Transition temperatures,  $T_m$  (°C) for hairpin probes with ssDNA targets.  $T^L$  and  $^{Me}C^L$  are locked nucleotides of thymine and 5-methylcytosine, respectively.

15 **Figure 35:** **A)** transition curves of the non-intercalating pseudonucleotide comprising probes **B)** Two LNA probes comprising one intercalating pseudonucleotide together with the unmodified reference duplex. **C)** LNA probes comprising one or two intercalating pseudonucleotide together with the unmodified reference duplex. **D)** A non-intercalating pseudonucleotide comprising LNA probes and two probes comprising

20 one intercalating pseudonucleotide together with homologically complementary DNA probe all hybridized to a target sequence comprising one intercalating pseudonucleotide..

**Figure 36: Scheme 1.** Schematic presentation of the conformations formed by  $T_4$ -LNA oligonucleotides at transition temperature.

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**Figure 37:** Synthesis of 1'-aza pyrenmethyl pseudonucleotide

**Figure 38:** Sequences and hybridisation data of synthesized ODNs in DNA/DNA(RNA) duplexes

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**Figure 39:** Hybridisation data for DNA Three-Way Junction

**Figure 40:** illustrates a beacon primer

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**Figure 41:** - illustrates a PCR quantification strategy using beacon primers

**Figure 42:** illustrates complete complementarity and mismatch/excimer formation

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## Detailed description of the invention

### Nucleic acids

10 The term "nucleic acid" covers the naturally occurring nucleic acids, DNA and RNA, including naturally occurring derivatives of DNA and RNA such as but not limited to methylated DNA, DNA containing adducts and RNA covalently bound to proteins. The term "nucleic acid analogues" covers synthetic derivatives and analogues of the naturally occurring nucleic acids, DNA and RNA. Synthetic analogues comprise one  
15 or more nucleotide analogues. The term "nucleotide analogue" comprises all nucleotide analogues capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing (see herein below), essentially like naturally occurring nucleotides.

20 Hence the terms "nucleic acids" or "nucleic acid analogues" designates any molecule, which essentially consists of a plurality of nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. Intercalator pseudonucleotides are described in detail herein below. Nucleic acids or nucleic acid analogues according to the present invention may comprise more different nucleotides and nucleotide  
25 analogues with different backbone monomer units (see herein below).

Preferably, single strands of nucleic acids or nucleic acid analogues according to the present invention are capable of hybridising with a substantially complementary single stranded nucleic acid and/or nucleic acid analogue to form a double stranded  
30 nucleic acid or nucleic acid analogue. More preferably such a double stranded analogue is capable of forming a double helix. Preferably, the double helix is formed due to hydrogen bonding, more preferably, the double helix is a double helix se-

lected from the group consisting of double helices of A form, B form, Z form and intermediates thereof.

Hence, nucleic acids and nucleic acid analogues according to the present invention includes; but is not limited to the kind of nucleic acids and/or nucleic acid analogues selected from DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA,  $\alpha$ -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA,  $\beta$ -D-Ribopyranosyl-NA,  $\alpha$ -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA,  $\alpha$ -L-RNA,  $\alpha$ -D-RNA,  $\beta$ -D-RNA and mixtures thereof and hybrids thereof, as well as phosphorous atom modifications thereof, such as but not limited to phosphorothioates, methyl phospholates, phosphoramidates, phosphorodithiates, phosphorosele-noates, phosphotriesters and phosphoboranoates. In addition non-phosphorous containing compounds may be used for linking to nucleotides such as but not limited to methyliminomethyl, formacetate, thioformacetate and linking groups comprising amides. In particular nucleic acids and nucleic acid analogues may comprise one or more intercalator pseudonucleotides according to the present invention.

Within this context "mixture" is meant to cover a nucleic acid or nucleic acid analogue strand comprising different kinds of nucleotides or nucleotide analogues. Furthermore, within this context, "hybrid" is meant to cover nucleic acids or nucleic acid analogues comprising one strand which comprises nucleotides or nucleotide analogues with one or more kinds of backbone and another strand which comprises nucleotides or nucleotide analogues with different kinds of backbone. By the term "duplex" is meant the hybridisation product of two strands of nucleic acids and/or nucleic acid analogues, wherein the strands preferably are of the same kind of nucleic acids and/or nucleic acid analogues.

By HNA is meant nucleic acids as for example described by Van Aetschot et al., 1995. By MNA is meant nucleic acids as described by Hossain et al, 1998. ANA refers to nucleic acids described by Allert et al, 1999. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. More preferably LNA is a nucleic acid as described in Singh et al, 1998, Koshkin et al, 1998 or Obika et al.,

1997. PNA refers to peptide nucleic acids as for example described by Nielsen et al., 1991.

5 The term nucleotide designates the building blocks of nucleic acids or nucleic acid analogues and the term nucleotide covers naturally occurring nucleotides and derivatives thereof as well as nucleotides capable of performing essentially the same functions as naturally occurring nucleotides and derivatives thereof. Naturally occurring nucleotides comprise deoxyribonucleotides comprising one of the four nucleobases adenine (A), thymine (T), guanine (G) or cytosine (C), and ribonucleotides  
10 comprising one of the four nucleobases adenine (A), uracil (U), guanine (G) or cytosine (C).

Nucleotide analogues may be any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing.

15 Non-naturally occurring nucleotides according to the present invention includes, but is not limited to the nucleotides selected from PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA,  $\alpha$ -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA,  $\beta$ -D-Ribopyranosyl-NA,  $\alpha$ -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA,  $\alpha$ -L-RNA, and  $\alpha$ -D-RNA,  $\beta$ -D-RNA  
20

The function of nucleotides and nucleotide analogues according to the present invention is to be able to interact specifically with complementary nucleotides via hydrogen bonding of the nucleobases of said complementary nucleotides as well as to be able to be incorporated into a nucleic acid or nucleic acid analogue. Naturally occurring nucleotides, as well as some nucleotide analogues are capable of being enzymatically incorporated into a nucleic acid or nucleic acid analogue, for example  
25 by RNA or DNA polymerases, however nucleotides or nucleotide analogues may also be chemically incorporated into a nucleic acid or nucleic acid analogue.  
30

Furthermore nucleic acids or nucleic acid analogues may be prepared by coupling two smaller nucleic acids or nucleic acid analogues to another, for example this may  
35 be done enzymatically by ligases or it may be done chemically.

Nucleotides or nucleotide analogues comprise a backbone monomer unit and a nucleobase. The nucleobase may be a naturally occurring nucleobase or a derivative thereof or an analogue thereof capable of performing essentially the same function.

5 The function of a nucleobase is to be capable of associating specifically with one or more other nucleobases via hydrogen bonds. Thus it is an important feature of a nucleobase that it can only form stable hydrogen bonds with one or a few other nucleobases, but that it can not form stable hydrogen bonds with most other nucleobases usually including itself. The specific interaction of one nucleobase with another nucleobase is generally termed "base-pairing".

Base pairing results in a specific hybridisation between predetermined and complementary nucleotides. Complementary nucleotides according to the present invention are nucleotides that comprise nucleobases that are capable of base-pairing.

15

Of the naturally occurring nucleobases adenine (A) pairs with thymine (T) or uracil (U); and guanine (G) pairs with cytosine (C). Accordingly, e.g. a nucleotide comprising A is complementary to a nucleotide comprising either T or U, and a nucleotide comprising G is complementary to a nucleotide comprising C.

20

Nucleotides according to the present invention may further be derivatised to comprise an appended molecular entity. The nucleotides can be derivatised on the nucleobases or on the backbone monomer unit. Preferred sites of derivatisation on the bases include the 8-position of adenine, the 5-position of uracil, the 5- or 6-position of cytosine, and the 7-position of guanine. The heterocyclic modifications can be grouped into three structural classes: Enhanced base stacking, additional hydrogen bonding and the combination of these. Modifications that enhance base stacking by expanding the  $\pi$ -electron cloud of planar systems are represented by conjugated, lipophilic modifications in the 5-position of pyrimidines and the 7-position of 7-deaza-purines. Substitutions in the 5-position of pyrimidines modifications include propynes, hexynes, thiazoles and simply a methyl group; and substituents in the 7-position of 7-deaza purines include iodo, propynyl, and cyano groups. It is also possible to modify the 5-position of cytosine from propynes to five-membered heterocycles and to tricyclic fused systems, which emanate from the 4- and 5-position (cytosine clamps). A second type of heterocycle modification is

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represented by the 2-amino-adenine where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the three hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified adenine and the tricyclic cytosine analog having an ethoxyamino functional group of heteroduplexes. Furthermore, N2-modified 2-amino adenine modified oligonucleotides are among commonly modifications. Preferred sites of derivatisation on ribose or deoxyribose moieties are modifications of nonconnecting carbon positions C-2' and C-4', modifications of connecting carbons C-1', C-3' and C-5', replacement of sugar oxygen, O-4', Anhydro sugar modifications (conformational restricted), cyclosugar modifications (conformational restricted), ribofuranosyl ring size change, connection sites – sugar to sugar, (C-3' to C-5'/ C-2' to C-5'), hetero-atom ring – modified sugars and combinations of above modifications.. However, other sites may be derivatised, as long as the overall base pairing specificity of a nucleic acid or nucleic acid analogue is not disrupted. Finally, when the backbone monomer unit comprises a phosphate group, the phosphates of some backbone monomer units may be derivatised.

Oligonucleotide or oligonucleotide analogue as used herein are molecules essentially consisting of a sequence of nucleotides and/or nucleotide analogues and/or intercalator pseudo-nucleotides. Preferably oligonucleotide or oligonucleotide analogue comprises 3-200, 5-100, 10-50 individual nucleotides and/or nucleotide analogues and/or intercalator pseudo-nucleotides, as defined above.

#### Target nucleic acids

A target nucleic acid or target nucleic acid analogue sequence refers to a nucleotide or nucleotide analogue sequence which comprise one or more sites/sequences for hybridisation of one or more oligonucleotide(s) and/or oligonucleotide analogue(s), for example primers or probes. Target sequences may be found in any nucleic acid or nucleic acid analogue including, but not limited too, other probes, RNA, genomic DNA, plasmid DNA, cDNA and can for example comprise a wild-type or mutant gene sequence or a regulatory sequence thereof or an amplified nucleic acid sequence, for example as when amplified by PCR. A target sequence may be of any length. The site addressed may or may not be one contiguous sequence. For example said site may be composed of two or more contiguous subsequences

separated by any number of nucleotides and/or nucleotide analogues. Preferentially the total length of the site addressed, composed by all subsequences on that particular target nucleic acid or target nucleic acid analogue, by said oligonucleotide and/or oligonucleotide analogue, typically is less than 100 nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides.

#### Homologous nucleic acids

Nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are said to be homologously complementary, when they are capable of hybridising. Preferably homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are capable of hybridising under low stringency conditions, more preferably homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are capable of hybridising under medium stringency conditions, more preferably homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are capable of hybridising under high stringency conditions.

High stringency conditions as used herein shall denote stringency as in comparison to, or at least as stringent as, what is normally applied in connection with Southern blotting and hybridisation as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include steps of prehybridization and hybridization. Such steps are normally performed using solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA (incubation for 18 hrs at 42°C), followed by washings with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described by Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), which is incorporated herein by reference.

Medium stringency conditions as used herein shall denote hybridisation in a buffer containing 1 mM EDTA, 10mM Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O, 140 mM NaCl, at pH 7.0, or a buffer similar to this having approximately the same impact on hybridization stringency. Preferably, around 1,5 µM of each nucleic acid or nucleic acid analogue strand is



provided. Alternatively medium stringency may denote hybridisation in a buffer containing 50 mM KCl, 10 mM TRIS-HCl (pH 9,0), 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>.

5 Low stringency conditions according to the present invention denote hybridisation in a buffer constituting 1 M NaCl, 10 mM Na<sub>3</sub>PO<sub>4</sub> at pH 7,0, or a buffer similar to this having approximately the same impact on hybridization stringency.

10 Alternatively, homologously complementary nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues are nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotide analogues substantially complementary to each other over a given sequence, such as more than 70% complementary, for example more than 75% complementary, such as more than 80% complementary, for example more than 85% complementary, such as more than 90% complementary, for example more than 92% complementary, such as more than 94% complementary, for example more than 95% complementary, such as more than 96% complementary, for example more than 97% complementary.

20 Preferably said given sequence is at least 4 nucleotides long, for example at least 10 nucleotides, such as at least 15 nucleotides, for example at least 20 nucleotides, such as at least 25 nucleotides, for example at least 30 nucleotides, such as between 10 and 500 nucleotides, for example between 4 and 100 nucleotides long, such as between 10 and 50 nucleotides long. More preferably homologously complementary oligonucleotides or oligonucleotide analogues are substantially homologously complementary over their entire length.

25

#### Specificity of hybridisation

30 The specificity of hybridisation of nucleic acids and/or nucleic acid analogues and/or oligonucleotides and/or oligonucleotide analogues refers to the ability of which said hybridisation event distinguishes between homologously complementary hybridisation partners according to their sequence differences under given stringency conditions. Often it is the intention to target only one particular sequence (the target sequence) in a mixture of nucleic acids and/or nucleic acid analogues and/or oligonucleotides and/or oligonucleotide analogues and to avoid hybridization to other sequences even though they have strong similarity to said target sequence. Some-

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times only one or few nucleotides differ among target and non-target sequences in the sequence-region used for hybridization.

5 High specificity in hybridisation as used herein denotes hybridisation under high stringency conditions at which an oligonucleotide or oligonucleotide analogue will hybridise with a homologous target sequence significantly better than to a nearly identical sequence differing only from said target sequence by one or few base-substitutions.

10 Discrimination

Discrimination refers to the ability of oligonucleotides and/or oligonucleotide analogues, in a sequence-independent manner, to hybridise preferentially with either RNA or DNA. Accordingly, the melting temperature of a hybrid consisting of oligonucleotide and/or oligonucleotide analogue and a homologously complementary RNA (RNA hybrid) is either significantly higher or lower than the melting temperature of a hybrid between said oligonucleotide and/or oligonucleotide analogue and a homologously complementary DNA (DNA hybrid).

20 RNA-like and DNA-like

RNA-like refers to nucleic acid analogues or oligonucleotide analogues behaving like RNA with respect to hybridisation to homologously complementary oligonucleotides and/or oligonucleotide analogues comprising at least one internal pseudonucleotide. Accordingly, RNA-like nucleic acid analogues or oligonucleotide analogues can be functionally categorized on the basis of their ability to hybridise with oligonucleotides and/or oligonucleotide analogues able to discriminate between RNA and DNA. Preferentially, said oligonucleotide analogues able to discriminate between RNA and DNA comprises one or more internally positioned pseudonucleotide intercalators and consequently, said oligonucleotide analogue comprising pseudonucleotide intercalators will preferentially not hybridise to said RNA-like nucleic acid analogues or oligonucleotide analogues.

Examples of RNA-like molecules are RNA, 2'-O-methyl RNA, LNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-LNA, 2'-R-RNA, 2'-OR-RNA, and mixtures thereof.

5 Likewise, DNA-like refers to nucleic acid analogues or oligonucleotide analogues behaving like DNA with respect to hybridisation to homologously complementary nucleic acids and/or nucleic acid analogues. Accordingly, DNA-like nucleic acids or nucleic acid analogues can be functionally categorized on the basis of their ability to hybridise with oligonucleotides or oligonucleotide analogues able to discriminate between RNA and DNA. Preferentially, said oligonucleotides or oligonucleotide analogues able to discriminate between RNA and DNA comprises one or more internally positioned pseudonucleotide intercalators, and consequently, said oligonucleotide analogue comprising pseudonucleotide intercalators will preferentially hybridise to said DNA-like nucleic acid analogues or oligonucleotide analogues.

15 Examples of DNA-like molecules is DNA and INA (Christensen, 2002. Intercalating nucleic acids containing insertions of 1-O-(1-pyrenylmethyl)glycerol: stabilisation of dsDNA and discrimination of DNA over RNA. Nucl. Acids. Res. 2002 30: 4918-4925).

20

### Cross-hybridisation

25 The term cross-hybridisation covers unattended hybridisation between at least two nucleic acids and/or nucleic acid analogues, i.e. cross-hybridisation may also be denoted intermolecular hybridisation. Hence the term cross-hybridization may be used to describe the hybridisation of for example a nucleic acid probe or nucleic acid analogue probe sequence to other nucleic acid sequences and/or nucleic acid analogue sequences than its intended target sequence.

30

Often cross-hybridization occurs between a probe and one or more homologously complementary non-target sequences, even though these have a lower degree of complementarity than the probe and its complementary target sequence. This unwanted effect could be due to a large excess of probe over target and/or fast an-

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nealing kinetics. Cross-hybridization also occurs by hydrogen bonding between few nucleobase pairs, e.g. between primers in a PCR reaction, resulting in primer dimer formation and/ or formation of unspecific PCR products.

- 5 Especially nucleic acids comprising one or more nucleotide analogues with high affinity for nucleotide analogues of the same type tend to form dimer or higher order complexes based on base pairing. Especially probes comprising nucleotide analogues such as, but not limited to, DNA, RNA, 2'-O-methyl RNA, PNA, HNA, MNA, ANA, LNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-  
10 LNA, 2'-R-RNA, 2'-OR-RNA, and mixtures thereof generally have a high affinity for hybridising to other oligonucleotide analogues comprising backbone monomer units of the same type. Hence even though individual probe molecules only have a low degree of complementarity, they tend to hybridise.

#### Self-hybridisation

- 15 The term self-hybridisation covers the process wherein a nucleic acid or nucleic acid analogue molecule anneals to itself by folding back on itself, generating a secondary structure like for example a hairpin structure, i.e. self-hybridisation may also be defined as intramolecular hybridisation. In most applications it is of importance to avoid  
20 self-hybridization. The generation of said secondary structures may inhibit hybridisation with desired nucleic acid target sequences. This is undesired in most assays for example when the nucleic acid or nucleic acid analogue is used as primer in PCR reactions or as fluorophore/ quencher labeled probe for exonuclease assays. In both assays self-hybridisation will inhibit hybridization to the target nucleic acid  
25 and additionally the degree of fluorophore quenching in the exonuclease assay is lowered.

- Especially nucleic acids comprising one or more nucleotide analogues with high affinity for nucleotide analogues of the same type tend to self-hybridise. Especially  
30 probes comprising nucleotide analogues such as, but not limited to, DNA, RNA, 2'-O-methyl RNA, PNA, HNA, MNA, ANA, LNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-LNA, 2'-R-RNA, 2'-OR-RNA generally have a high affinity for self-hybridising. Hence even though individual probe molecules only have a low degree of self-complementarity they tend to self-hybridise.

Melting temperature

Melting of nucleic acids refer to thermal separation of the two strands of a double-stranded nucleic acid molecule.

5

The melting temperature ( $T_m$ ) denotes the temperature in degrees centigrade at which 50% helical (hybridised) versus coil (unhybridised) forms are present.

10

A high melting temperature is indicative of a stable complex and accordingly of a high affinity between the individual strands. Vice versa a low melting temperature is indicative of a relatively low affinity between the individual strands. Accordingly, usually strong hydrogen bonding between the two strands results in a high melting temperature.

15

Furthermore, as disclosed by the present invention, intercalation of an intercalator between nucleobases of a double stranded nucleic acid may also stabilise double stranded nucleic acids and accordingly result in a higher melting temperature.

20

In addition the melting temperature is dependent on the physical/chemical state of the surroundings. For example the melting temperature is dependent on salt concentration and pH.

25

The melting temperature may be determined by a number of assays, for example it may be determined by using the UV spectrum to determine the formation and breakdown (melting) of hybridisation.

Backbone monomer unit

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The backbone monomer unit of a nucleotide or a nucleotide analogue or an intercalator pseudonucleotide according to the present invention is the part of the nucleotide, which is involved in incorporation of the nucleotide or nucleotide analogue or intercalator pseudonucleotide into the backbone of a nucleic acid or a nucleic acid analogue. Any suitable backbone monomer unit may be employed with the present invention.

In particular the backbone monomer unit of intercalator pseudonucleotides according to the present invention may be selected from the backbone monomer units mentioned herein below.

- 5 The backbone monomer unit comprises the part of a nucleotide or nucleotide analogue or intercalator pseudonucleotide that may be incorporated into the backbone of an oligonucleotide or an oligonucleotide analogue. In addition, the backbone monomer unit may comprise one or more leaving groups, protecting groups and/or reactive groups, which may be removed or changed in any way during synthesis or  
10 subsequent to synthesis of an oligonucleotide or oligonucleotide analogue comprising said backbone monomer unit.

It is important to note that the term backbone monomer unit according to the present invention only includes the backbone monomer unit per se and it does not include  
15 for example a linker connecting a backbone monomer unit to an intercalator. Hence, the intercalator as well as the linker is not part of the backbone monomer unit.

Accordingly, backbone monomer units only include atoms, wherein the monomer is incorporated into a sequence, are selected from the group consisting of

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- a) atoms which are capable of forming a linkage to the backbone monomer unit of a neighboring nucleotide; or
- b) atoms which at least at two sites are connected to other atoms of the backbone monomer unit; or
- 25 c) atoms which at one site is connected to the backbone monomer unit and otherwise is not connected with other atoms

More preferably, backbone monomer unit atoms are thus defined as the atoms involved in the direct linkage (shortest path) between the backbone Phosphor-atoms  
30 of neighbouring nucleotides, when the monomer is incorporated into a sequence, wherein the neighbouring nucleotides are naturally occurring nucleotides.

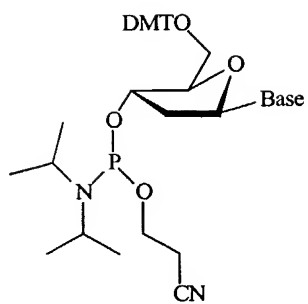
The backbone monomer unit may be any suitable backbone monomer unit. In one embodiment of the present invention, the backbone monomer unit may for example  
35 be selected from the group consisting of the backbone monomer units of DNA, RNA,

PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA,  $\alpha$ -L-Ribo-LNA,  $\alpha$ -L-Xylo-LNA,  $\beta$ -D-Xylo-LNA,  $\alpha$ -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA,  $\alpha$ -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA,  $\beta$ -D-Ribopyranosyl-NA,  $\alpha$ -L-Lyxopyranosyl-NA, 2'-R-RNA,  $\alpha$ -L-RNA or  $\alpha$ -D-RNA,  $\beta$ -D-RNA.

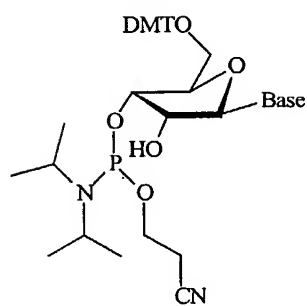
Below is depicted a range of different backbone monomer units of nucleotides and nucleotide analogues, and how they are connected to the nucleobases via linkers that are attached at one or two positions of the backbone monomer unit:

## Examples of oligomers of DNA, RNA &amp; PNA

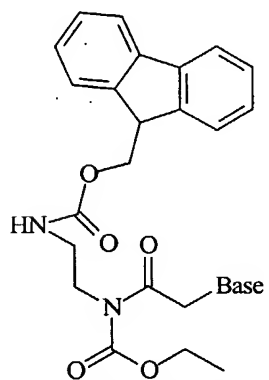
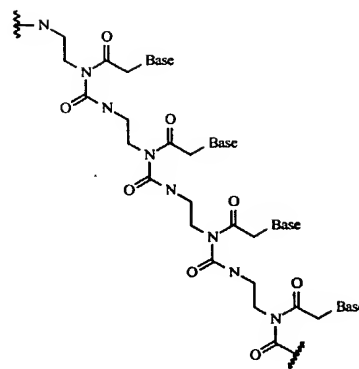
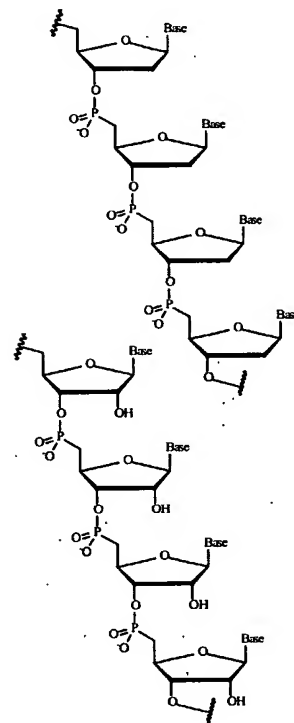
## DNA



## RNA

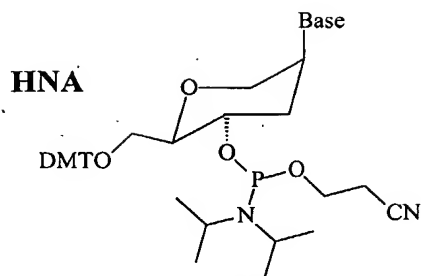


## PNA

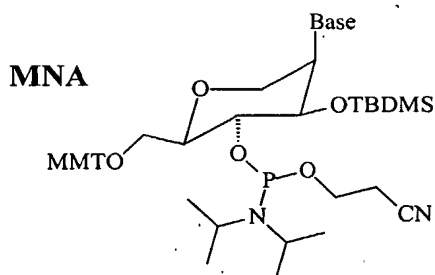
Ref. Nielsen, P. E. et al. *Science*, 1991, 254, 1497.



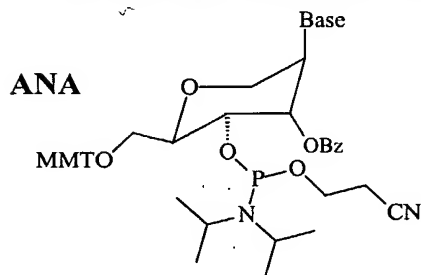
## Examples of oligomers of some analogues



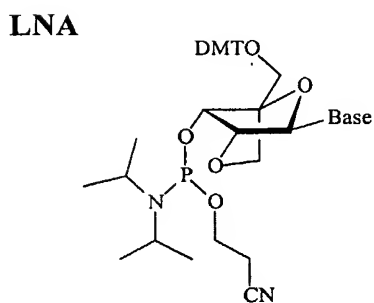
Ref. Van Aerschot, A. et al. *Angew. Chem. Int. Ed. Engl.*, 1995, 34, 1338-1339.



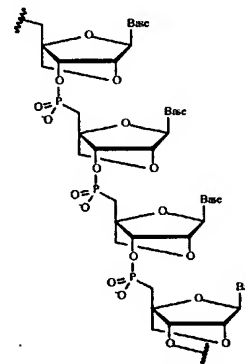
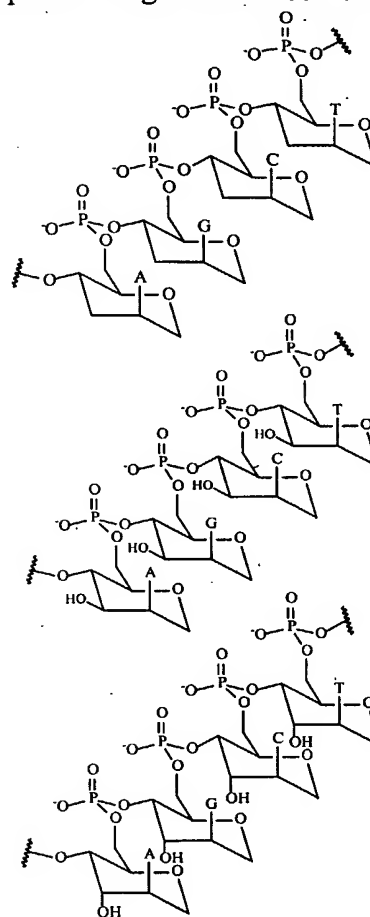
Ref. Hossain N. et al. *J. Org. Chem.*, 1998, 63, 1574-1582.



Ref. Allart, B. et al. *Chem. Eur. J.*, 1999, 5, 2424-2431.

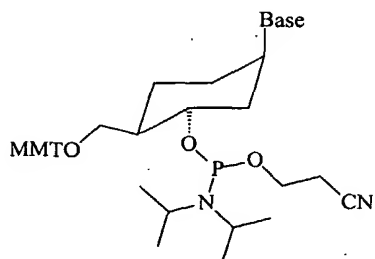


Ref. Singh, S. K. et al. *Chem. Commun.*, 1998, 455-456; Koshkin, A.A. et al. *Tetrahedron*, 1998, 54, 3607-3630; Obika, S. et al. *Tetrahedron Lett.*, 1997, 38, 8735-8738.



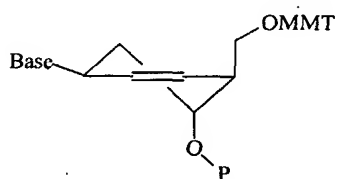
## Examples of oligomers of some analogues

## Cyclohexanyl-NA (CNA)



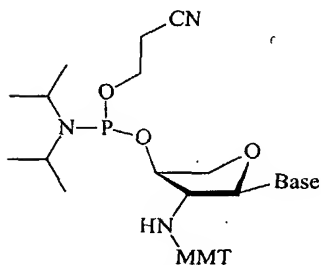
Ref: Maurinsh, Y.; et al. Chem. Eur. J., 1999, 2139-2150.

## Cyclohexenyl-NA (CeNA)



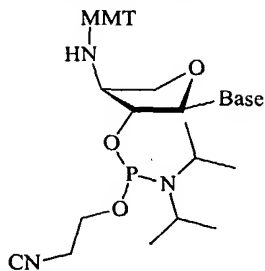
Ref: Wang, J.; et al. J. Am. Chem. Soc, 2000, 8595-8602.

## (2'-NH)-TNA

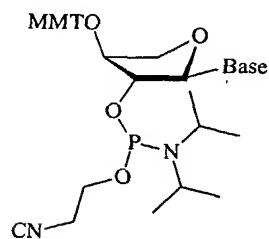


Ref: Wu, X. et al., Org. Lett., 2002, 4, 1279-1282

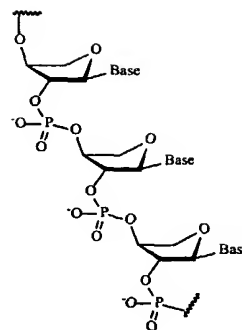
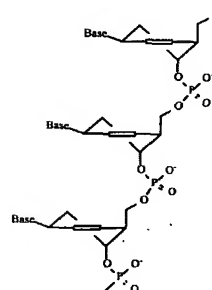
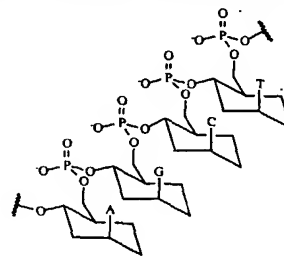
## (3'-NH)-TNA



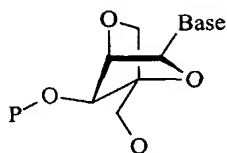
## TNA



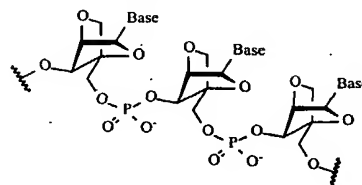
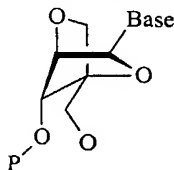
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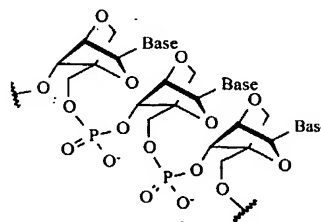
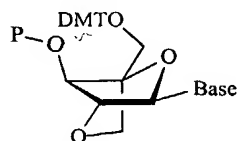
## Section of a nucleic acid of the respective analogues

 $\alpha$ -L-Ribo-LNA

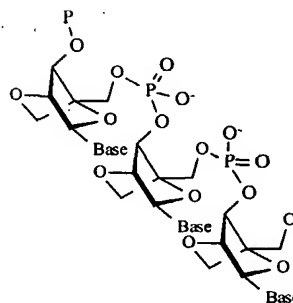
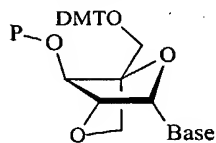
Ref: Rajwanshi, V. K. et al. Chem. Commun., 1999, 1395-1396.

 $\alpha$ -L-Xylo-LNA

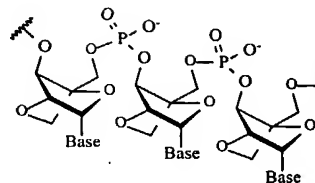
Ref: Rajwanshi, V. K et al. Angew. Chem. Int. Ed., 2000, 1656-1659.

 $\beta$ -D-Xylo-LNA

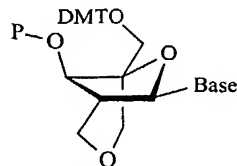
Ref: Rajwanshi, V. K. et al. Chem. Commun., 1999, 1395-1396.

 $\alpha$ -D-Ribo-LNA

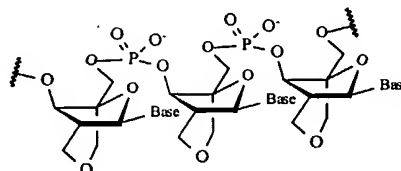
Ref: Rajwanshi, V. K et al. Angew. Chem. Int. Ed., 2000, 1656-1659.



## [3.2.1]-LNA

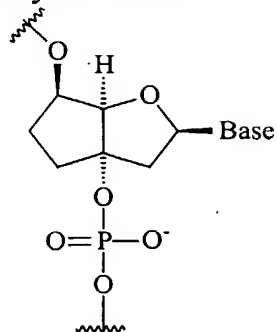


Ref: Wang, G.; et al. Tetrahedron, 1999, 7707-2724.

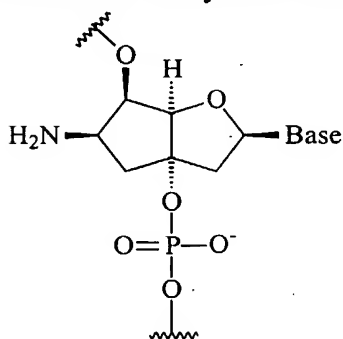


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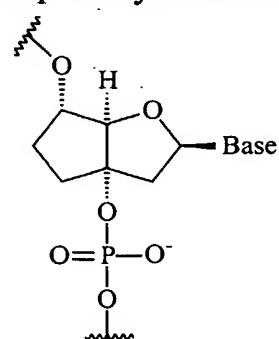
Bicyclo-DNA



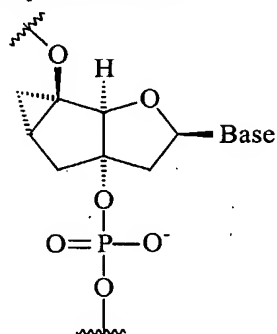
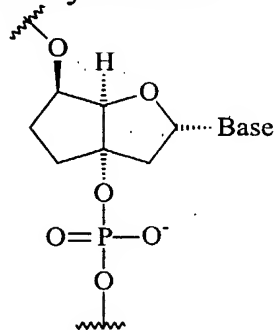
6'-Amino-Bicyclo-DNA



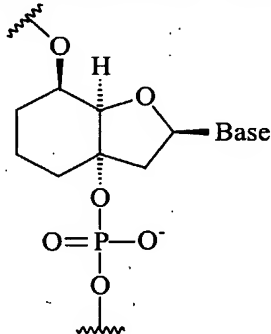
5'-epi-Bicyclo-DNA



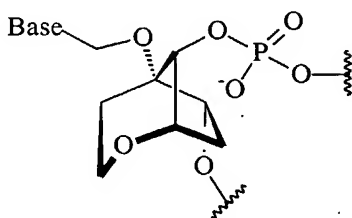
Tricyclo-DNA

 $\alpha$ -Bicyclo-DNA

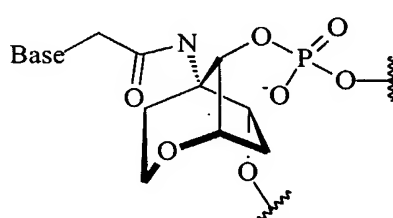
Bicyclo[4.3.0]-DNA



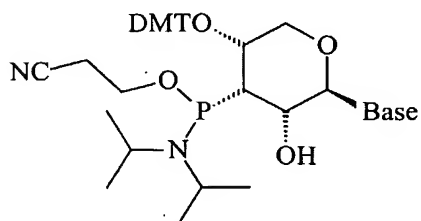
Bicyclo[3.2.1]-DNA



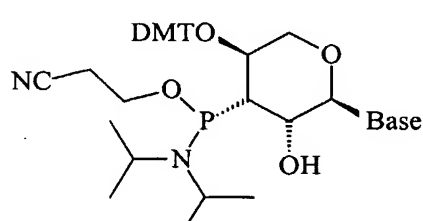
Bicyclo[3.2.1]amide-DNA



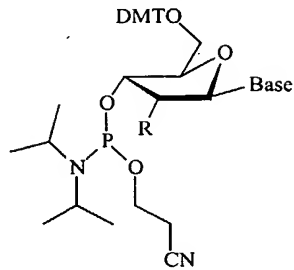
Ref: All of the Bicyclo-DNAs are reviewed in Leumann, C. J., Bioorg. Med. Chem., 2002, 841-854.

 $\beta$ -D-Ribopyranosyl-NA

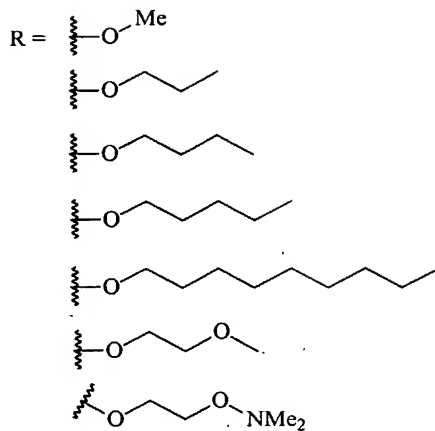
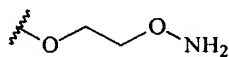
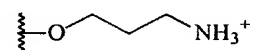
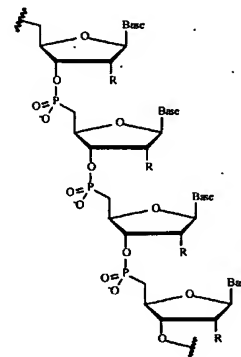
Ref: Reck, F. et al., Org. Lett. 1999, 1, 1531

 $\alpha$ -L-Lyxopyranosyl-NA

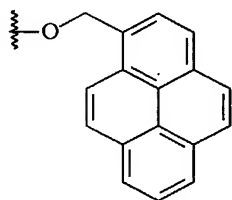
Ref: Reck, F. et al., Org. Lett. 1999, 1, 1531

**2'-R-RNA**

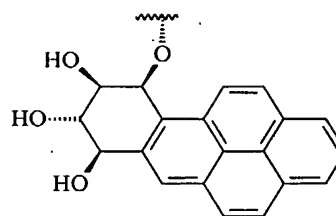
R = F

**General structure of 2'-modified oligomers**

Ref: Reviewed by Manoharan, M. Biochim. BioPhys. Acta, 1999, 117-130.

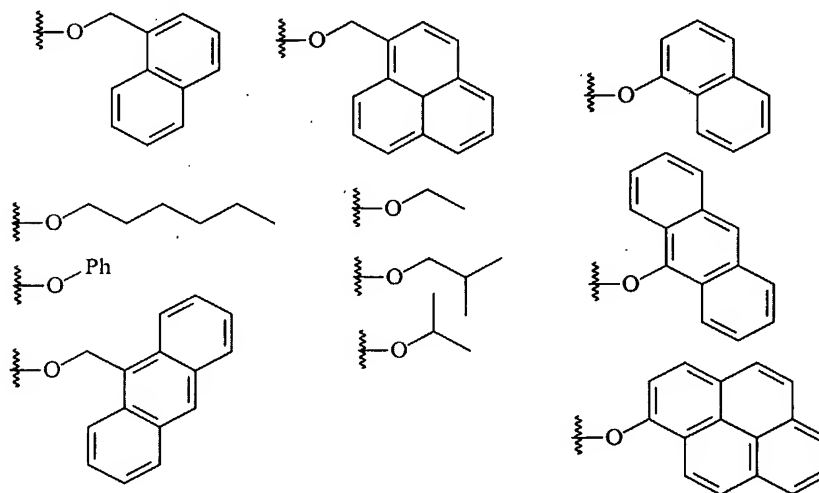


Ref: Yamana, K. et al., Tetrahedron Lett., 1991, 6347-6350.



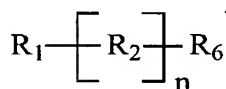
Ref: Sayer, J. et al., J. Org. Chem., 1991, 56, 20-29.

Examples of modifications that, to our knowledge, are not synthesised or published yet:



The backbone monomer unit of LNA (locked nucleic acid) is a sterically restricted DNA backbone monomer unit, which comprises an intramolecular bridge that restricts the usual conformational freedom of a DNA backbone monomer unit. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. Preferred LNA according to the present invention comprises a methyl linker connecting the 2'-O position to the 4'-C position, however other LNA's such as LNA's wherein the 2' oxy atom is replaced by either nitrogen or sulphur are also comprised within the present invention.

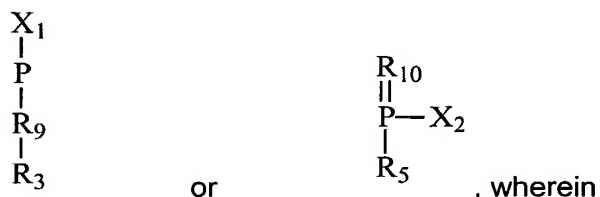
The backbone monomer unit of intercalator pseudonucleotides according to present invention preferably have the general structure before being incorporated into an oligonucleotide and/or nucleotide analogue:



wherein

$n = 1$  to 6, preferably  $n = 2$  to 6, more preferably  $n = 3$  to 6, more preferably  $n = 2$  to 5, more preferably  $n = 3$  to 5, more preferably  $n = 3$  to 4.

$R_1$  is a trivalent or pentavalent substituted phosphoratom, preferably  $R_1$  is



$R_2$  may individually be selected from an atom capable of forming at least two bonds, said atom optionally being individually substituted, preferably  $R_2$  is individually selected from O, S, N, C, P, optionally individually substituted. By the term "individually" is meant that  $R_2$  can represent one, two or more different groups in the same

molecule. The bonds between two  $R_2$  may be saturated or unsaturated or a part of a ring system or a combination thereof

5 Each  $R_2$  may individually be substituted with any suitable substituent, such as a substituent selected from H, lower alkyl, C2-6 alkenyl, C6-10 aryl, C7-11 arylmethyl, C2-7 acyloxymethyl, C3-8 alkoxycarbonyloxymethyl, C7-11 aryloxyloxymethyl, C3-8 S-acyl-2-thioethyl;

10 An "alkyl" group refers to an optionally substituted saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkyl of from 1 to 12 carbons, more preferably 1 to 6 carbons, more preferably 1 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

15 An "alkenyl" group refers to an optionally substituted hydrocarbon containing at least one double bond, including straight-chain, branched-chain, and cyclic alkenyl groups, all of which may be optionally substituted. Preferably, the alkenyl group has 2 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkenyl of from 2 to 12 carbons, more preferably 2 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

25 An "alkynyl" group refers to an optionally substituted unsaturated hydrocarbon containing at least one triple bond, including straight-chain, branched-chain, and cyclic alkynyl groups, all of which may be optionally substituted. Preferably, the alkynyl group has 2 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkynyl of from 2 to 12 carbons, more preferably 2 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

30 An "aryl" refers to an optionally substituted aromatic group having at least one ring with a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl, biaryl, and triaryl groups. Examples of aryl substitution substituents include alkyl,

alkenyl, alkynyl, aryl, amino, substituted amino, carboxy, hydroxy, alkoxy, nitro, sulfonyl, halogen, thiol and aryloxy.

5 A "carbocyclic aryl" refers to an aryl where all the atoms on the aromatic ring are carbon atoms. The carbon atoms are optionally substituted as described above for an aryl. Preferably, the carbocyclic aryl is an optionally substituted phenyl.

10 A "heterocyclic aryl" refers to an aryl having 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen. Examples of heterocyclic aryls include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, and imidazolyl. The heterocyclic aryl is optionally substituted as described above for an aryl.

The substituents on two or more  $R_2$  may alternatively join to form a ring system, such as any of the ring systems as defined above.

15 Preferably  $R_2$  is substituted with an atom or a group selected from H, methyl,  $R_4$ , hydroxyl, halogen, and amino, more preferably  $R_2$  is substituted with an atom or a group selected from H, methyl,  $R_4$ .

More preferably  $R_2$  is individually selected from O, S, NH, N(Me), N( $R_4$ ), C( $R_4$ )<sub>2</sub>, CH( $R_4$ ) or CH<sub>2</sub>, wherein  $R_4$  is as defined below,

20

$R_3$  = methyl, beta-cyanoethyl, p-nitrophenetyl, o-chlorophenyl, or p-chlorophenyl.

25  $R_4$  = lower alkyl, preferably lower alkyl such as methyl, ethyl, or isopropyl, or heterocyclic, such as morpholino, pyrrolidino, or 2,2,6,6-tetramethylpyrrolidino, wherein lower alkyl is defined as C<sub>1</sub> - C<sub>6</sub>, such as C<sub>1</sub> - C<sub>4</sub>.

$R_5$  = alkyl, alkoxy, aryl or H, with the proviso that  $R_5$  is H when  $X_2 = O^-$ , preferably  $R_5$  is selected from lower alkyl, lower alkoxy, aryloxy. In a preferred embodiment aryloxy is selected from phenyl, naphthyl or pyridine.

30  $R_6$  is a protecting group, selected from any suitable protecting groups. Preferably  $R_6$  is selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-tetrachloro-2,2-bis(p-methoxyphenyl)-ethan (DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups



mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably the protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting group may be 4, 4'-dimethoxytrityl (DMT).

5

$R_9$  is selected from O, S, N optionally substituted, preferably  $R_9$  is selected from O, S, NH, N(Me).

$R_{10}$  is selected from O, S, N, C, optionally substituted.

10

$X_1 = \text{Cl, Br, I, or } N(R_4)_2$

$X_2 = \text{Cl, Br, I, } N(R_4)_2, \text{ or } O^-$

15

As described above with respect to the substituents the backbone monomer unit can be acyclic or part of a ring system.

20

In one preferred embodiment of the present invention the backbone monomer unit of an intercalator pseudonucleotide is selected from the group consisting of acyclic backbone monomer units. Acyclic is meant to cover any backbone monomer unit, which does not comprise a ringstructure, for example the backbone monomer unit preferably does not comprise a ribose or a deoxyribose group.

25

In particular, it is preferred that the backbone monomer unit of an intercalator pseudonucleotide is an acyclic backbone monomer unit, which is capable of stabilising a bulge insertion (see herein below).

30

In another preferred embodiment the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from the group consisting of trivalent and pentavalent phosphorous atom such as a pentavalent phosphorous atom. More preferably the phosphate atom of the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from the group

35

consisting of, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups.

5 In particular it is preferred that the backbone monomer unit of an intercalator pseudonucleotide according to the present invention is selected from the group consisting of acyclic backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups.

10 Preferred backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidit groups are backbone monomer units, wherein the distance from at least one phosphor atom to at least one phosphor atom of a neighbouring nucleotide, not including the phosphor atoms, is at the most 6  
15 atoms long, for example 2, such as 3, for example 4, such as 5, for example 6 atoms long, when the backbone monomer unit is incorporated into a nucleic acid backbone.

20 The distance is measured as the direct linkage (i.e. the shortest path) as discussed above.

Preferably the backbone monomer unit is capable of being incorporated into a phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 5 atoms are separating the phosphor atom of the intercalator  
25 pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, more preferably 5 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, in both cases not including the phosphor atoms themselves.

30 Preferably the backbone monomer unit is capable of being incorporated into a phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 4 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, more preferably 4 atoms are separating the phosphor atom of the intercalator

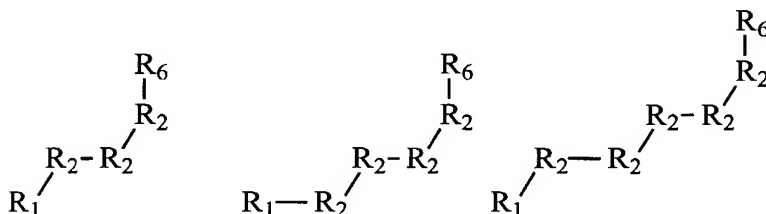
pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, in both cases not including the phosphor atoms themselves.

In a particularly preferred embodiment of the present invention the intercalator  
 5 pseudonucleotide comprises a backbone monomer unit that comprises a phosphoramidit and more preferably the backbone monomer unit comprises a trivalent phosphoramidit.

Suitable trivalent phosphoramidits are trivalent phosphoramidits that may be  
 10 incorporated into the backbone of a nucleic acid and/or a nucleic acid analogue. Usually, the amidit group per se may not be incorporated into the backbone of a nucleic acid, but rather the amidit group or part of the amidit group may serve as a leaving group and/ or protecting group. However, it is preferred that the backbone  
 15 monomer unit comprises a phosphoramidit group, because such a group may facilitate the incorporation of the backbone monomer unit into a nucleic acid backbone.

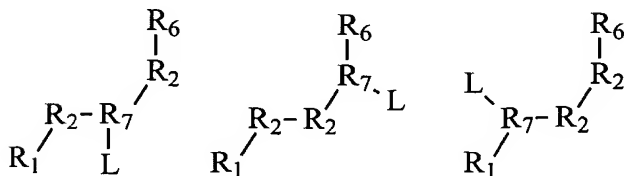
Preferably the acyclic backbone monomers may be selected from one of the general  
 structures depicted below:

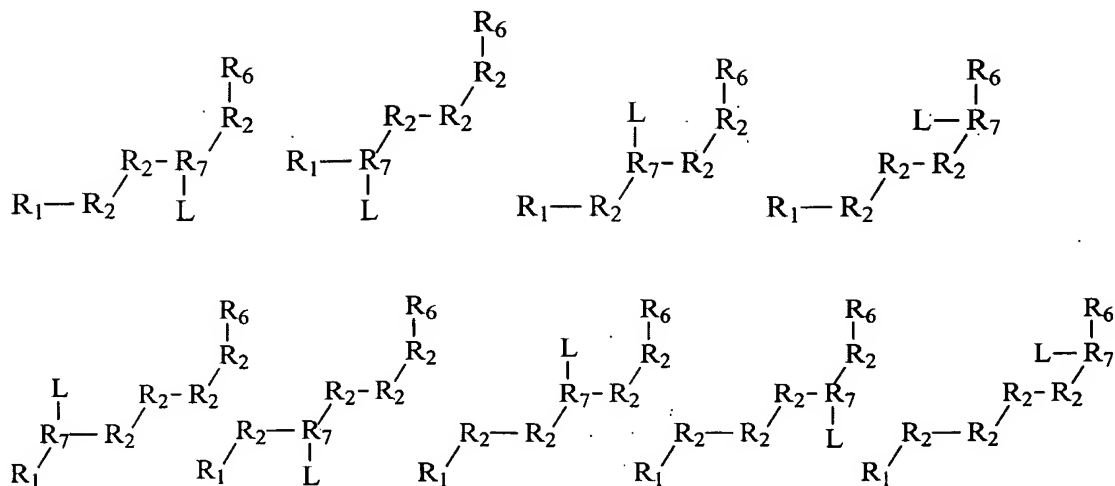
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wherein  $R_1$ ,  $R_2$  and  $R_6$  are as defined above.

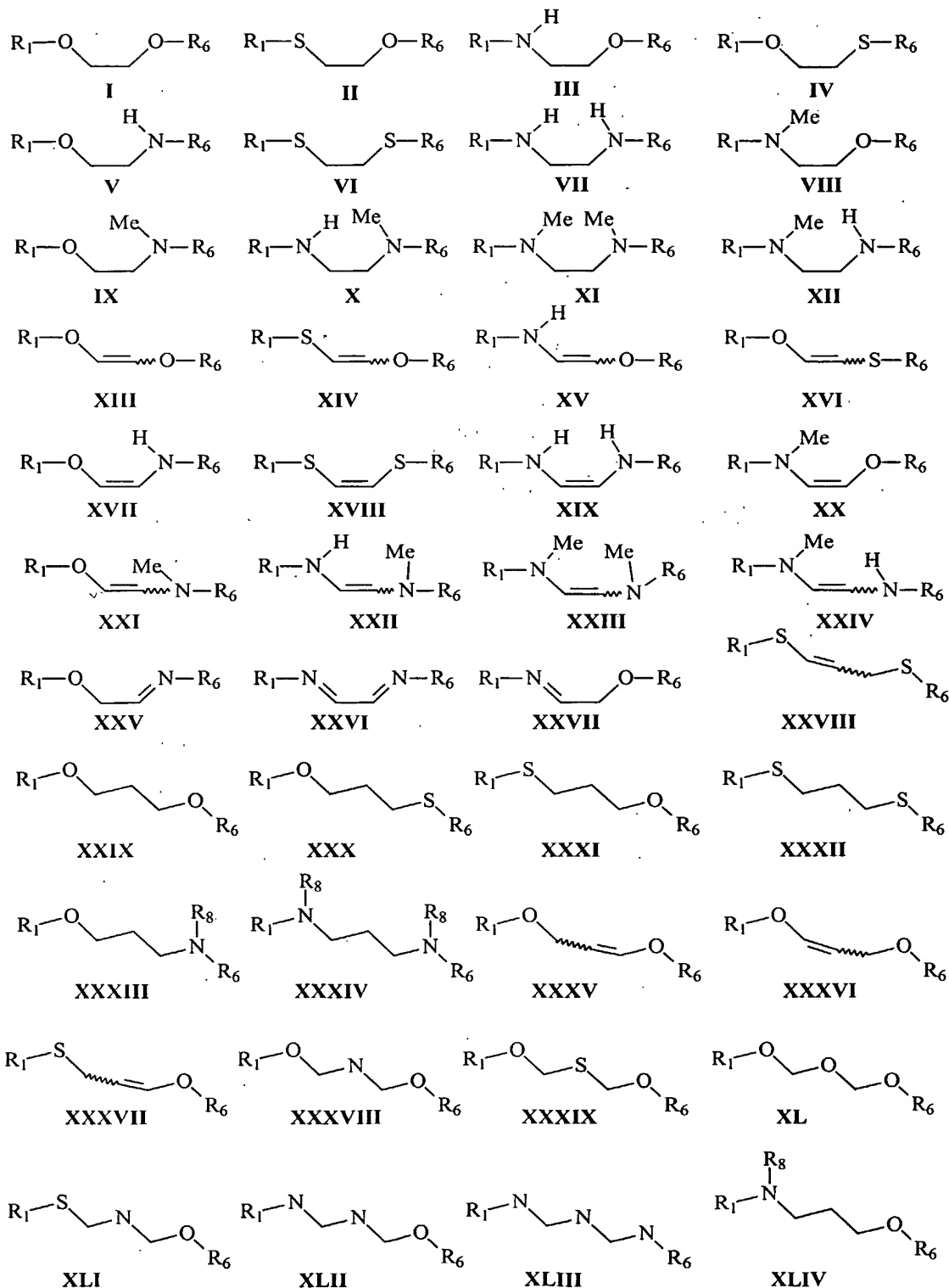
25 More preferably, the acyclic backbone monomer unit may be selected from the group depicted below:





5 wherein  $R_1$ ,  $R_2$  and  $R_6$  are as defined above, and  $R_7 = N$ , or  $CH$ , and  $L$  is a linker.

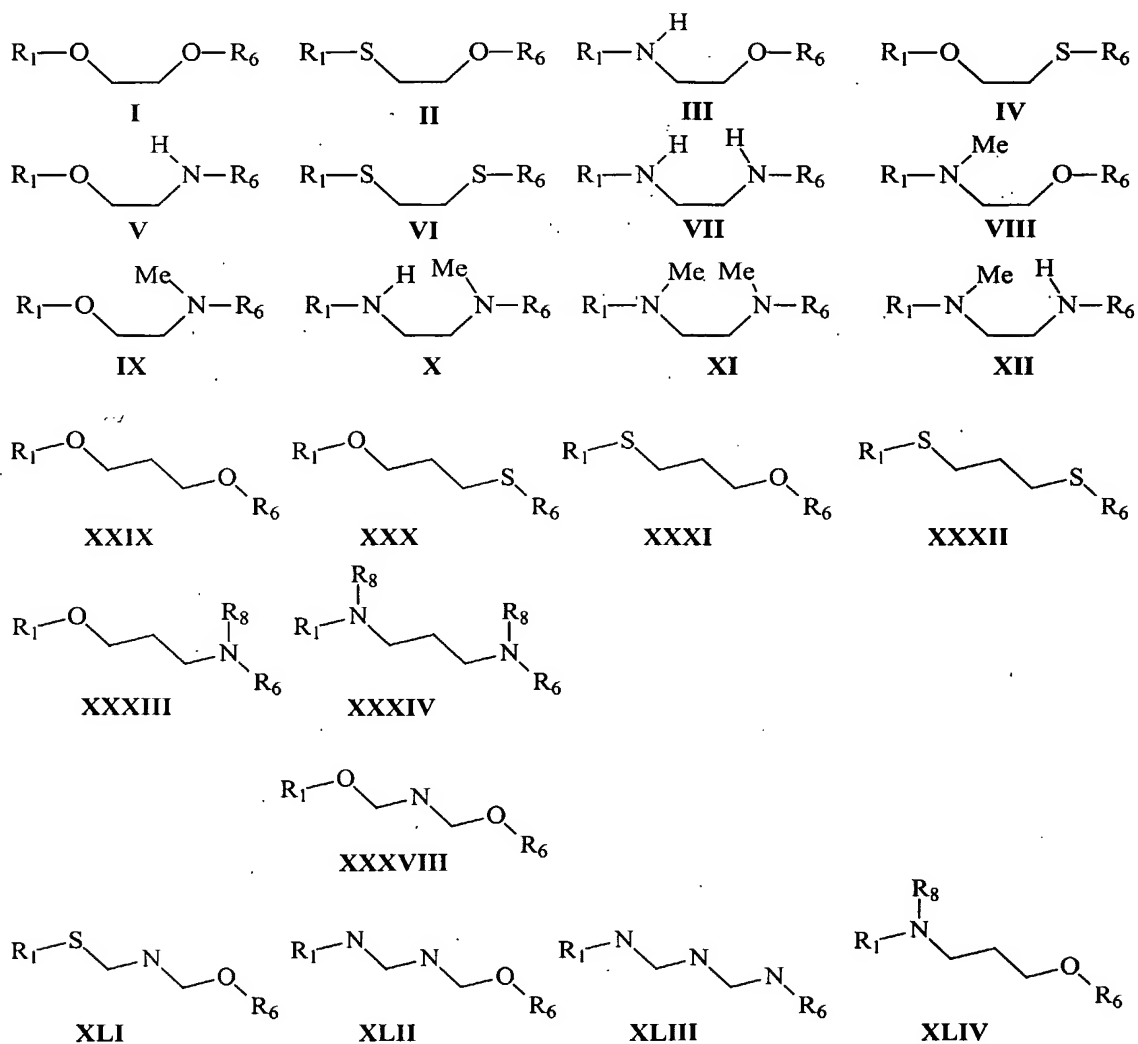
Below are specific examples of backbone monomer units numbered 1) to , wherein  $R_1$  and  $R_6$  are as defined above, and  $R_8$  may be  $R_4$  or  $H$ , optionally substituted.



Me denotes methyl

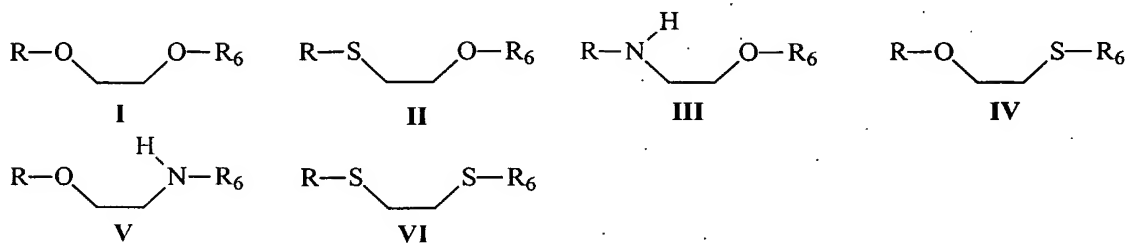
Even more preferable the backbone monomer unit including optional protecting groups may be selected from the group consisting of the structures I) to XLIV) as indicated herein below:

5



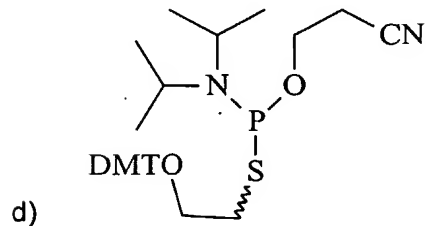
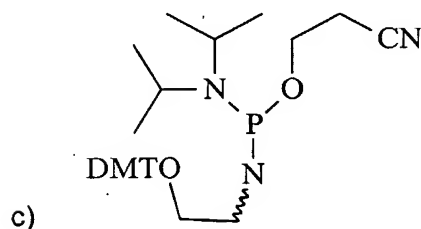
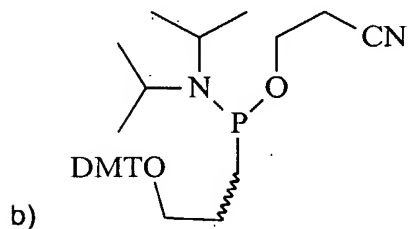
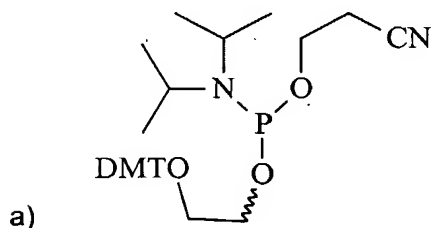
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Most preferred are the backbone monomer units selected from the group consisting of:

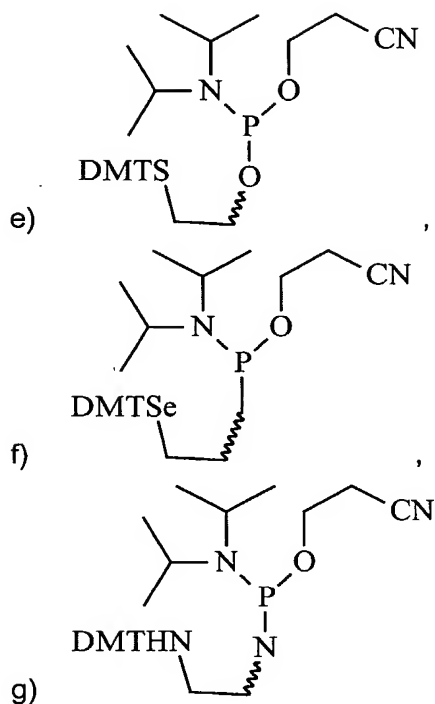


5

Preferably, the acyclic backbone monomer unit may be selected from the group consisting of the structures a) to g) as indicated below:



10



5

The backbone monomer unit of an intercalator pseudonucleotide which is inserted into an oligonucleotide or oligonucleotide analogue, according to the present invention may comprise a phosphodiester bond. Additionally, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention may comprise a pentavalent phosphoramidate. Preferably, the backbone monomer unit of an intercalator pseudonucleotide according to the present invention is an acyclic backbone monomer unit that may comprise a pentavalent phosphoramidate.

#### 15 Leaving group

The backbone monomer unit according to the present invention may comprise one or more leaving groups. Leaving groups are chemical groups, which are part of the backbone monomer unit when the intercalator pseudonucleotide or the nucleotide is a monomer, but which are no longer present in the molecule once the intercalator pseudonucleotide or the nucleotide has been incorporated into an oligonucleotide or oligonucleotide analogue.



5 The nature of a leaving group depends of the backbone monomer unit. For example, when the backbone monomer unit is a phosphor amidit, the leaving group, may for example be an diisopropylamine group. In general, when the backbone monomer unit is a phosphor amidit, a leaving group is attached to the phosphor atom for example in the form of diisopropylamine and said leaving group is removed upon coupling of the phosphor atom to a nucleophilic group, whereas the rest of the phosphate group, may become part of the nucleic acid or nucleic acid analogue backbone.

10 Reactive group

The backbone monomer units according to the present invention may furthermore comprise a reactive group which is capable of performing a chemical reaction with another nucleotide or oligonucleotide or nucleic acid or nucleic acid analogue to form a nucleic acid or nucleic acid analogue, which is one nucleotide longer than before the reaction.

15 Accordingly, when nucleotides are in their free form, i.e. not incorporated into a nucleic acid, they may comprise a reactive group capable of reacting with another nucleotide or a nucleic acid or nucleic acid analogue.

20 In preferred embodiments of the present invention said reactive group may be protected by a protecting group. Prior to said chemical reaction, said protection group may be removed. The protection group will thus not be a part of the newly formed nucleic acid or nucleic acid analogue.

25 Examples of reactive groups are nucleophiles such as the 5'-hydroxy group of DNA or RNA backbone monomer units.

Protecting group

30 The backbone monomer unit according to the present invention may also comprise a protecting group, which can be removed, and wherein removal of the protecting group allows for a chemical reaction between the intercalator pseudonucleotide and a nucleotide or nucleotide analogue or another intercalator pseudonucleotide.

In particular, a nucleotide monomer or nucleotide analogue monomer or intercalator pseudonucleotide monomer may comprise a protecting group, which is no longer present in the molecule once the nucleotide or nucleotide analogue or intercalator pseudonucleotide has been incorporated into a nucleic acid or nucleic acid analogue.

Furthermore, backbone monomer units may comprise protecting groups which may be present in the oligonucleotide or oligonucleotide analogue subsequent to incorporation of the nucleotide or nucleotide analogue or intercalator pseudonucleotide, but which may no longer be present after introduction of an additional nucleotide or nucleotide analogue to the oligonucleotide or oligonucleotide analogue or which may be removed after the synthesis of the entire oligonucleotide or oligonucleotide analogue.

The protecting group may be removed by a number of suitable techniques known to the person skilled in the art, however preferably, the protecting group may be removed by a treatment selected from the group consisting of acid treatment, thiophenol treatment and alkali treatment.

Preferred protecting groups according to the present invention, which may be used to protect the 5' end or the 5' end analogue of a backbone monomer unit may be selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-tetrachloro-2,2-bis(p-methoxyphenyl)-ethan (DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably the protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting group may be 4, 4'-dimethoxytrityl(DMT).

4, 4'-dimethoxytrityl(DMT) groups may be removed by acid treatment, for example by brief incubation (30 to 60 seconds sufficient) in 3% trichloroacetic acid or in 3% dichloroacetic acid in  $\text{CH}_2\text{Cl}_2$ .

Preferred protecting groups which may protect a phosphate or phosphoramidite group of a backbone monomer unit may for example be selected from the group consisting of methyl and 2-cyanoethyl. Methyl protecting groups may for example be removed by treatment with thiophenol or disodium 2-carbamoyl 2-cyanoethylene-1,1-dithiolate. 2-cyanoethyl-groups may be removed by alkali treatment, for example treatment with concentrated aqueous ammonia, a 1:1 mixture of aqueous methylamine and concentrated aqueous ammonia or with ammonia gas.

#### Intercalator

The term intercalator according to the present invention covers any molecular moiety comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid. Preferably an intercalator according to the present invention essentially consists of at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid or nucleic acid analogue.

Preferably, the intercalator comprises a chemical group selected from the group consisting of polyaromates and heteropolyaromates and even more preferably the intercalator essentially consists of a polyaromate or a heteropolyaromate. Most preferably the intercalator is selected from the group consisting of polyaromates and heteropolyaromates.

Polyaromates or heteropolyaromates according to the present invention may consist of any suitable number of rings, such as 1, for example 2, such as 3, for example 4, such as 5, for example 6, such as 7, for example 8, such as more than 8. Furthermore polyaromates or heteropolyaromates may be substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and amido.

In one preferred embodiment of the present invention the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that are capable of fluorescing.

In another more preferred embodiment of the present invention the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that are capable of forming excimers, exciplexes, fluorescence resonance energy transfer (FRET) or charged transfer complexes.

5

Accordingly, the intercalator may preferably be selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins, psoralens and any of the aforementioned intercalators substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and/or amido.

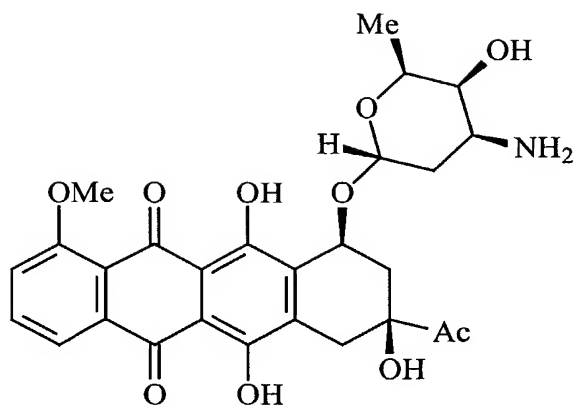
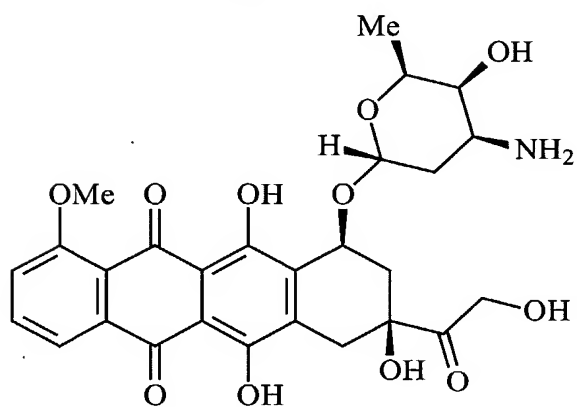
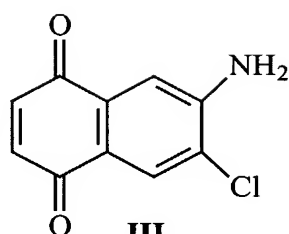
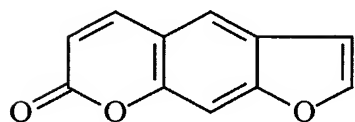
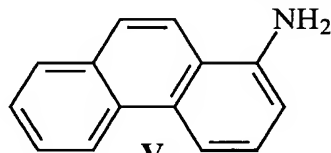
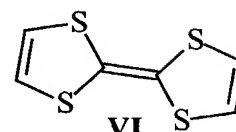
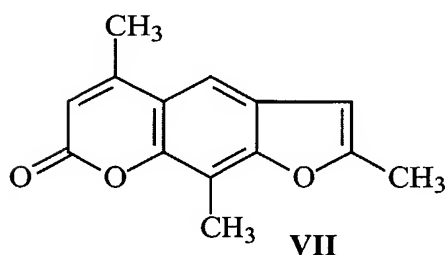
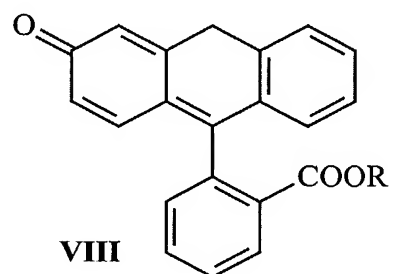
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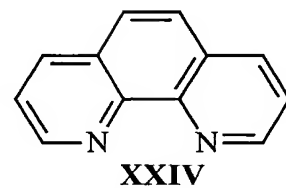
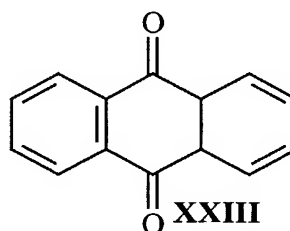
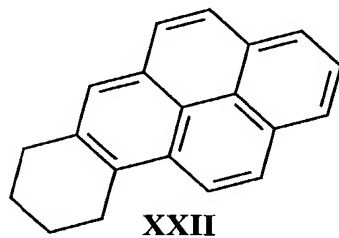
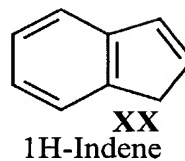
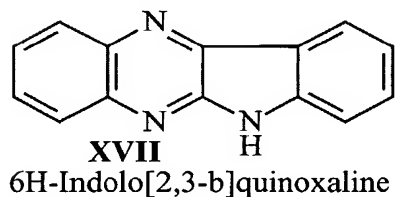
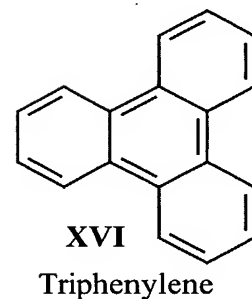
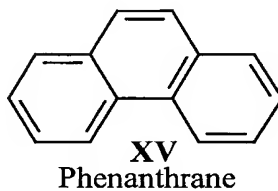
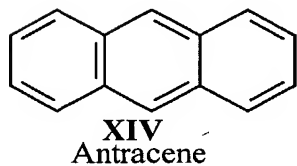
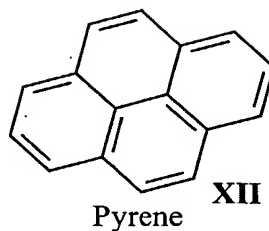
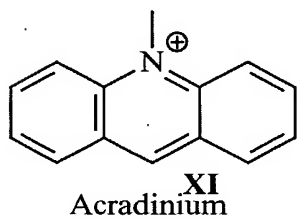
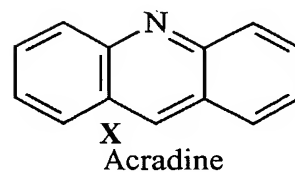
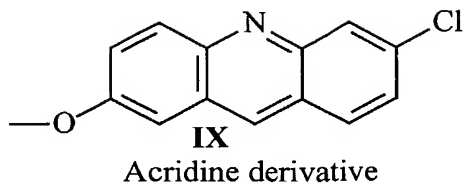
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Preferably, the intercalator is selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.

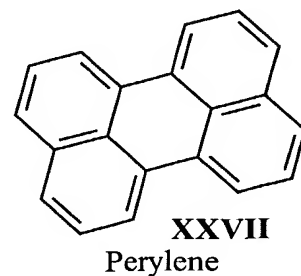
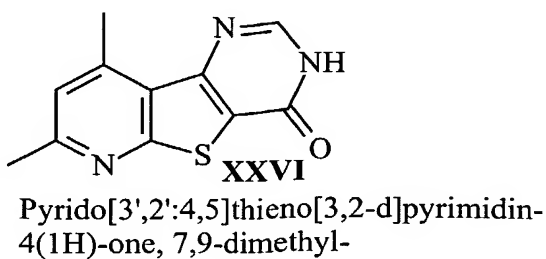
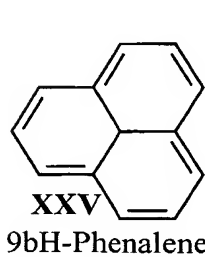
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More preferably the intercalator may be selected from the group of intercalators comprising one of the structures as indicated herein below:

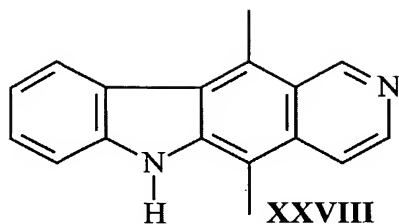
Daunomycin **I**Doxorubicin **II**Naphtoquinone **III**Psoralene **IV**Phenanthroline **V**1,3-Dithiole,  
2-(1,3-dithiol-2-ylidene)-  
**VI**Trimethylpsoralene **VII****VIII**  
Fluorescein derivative



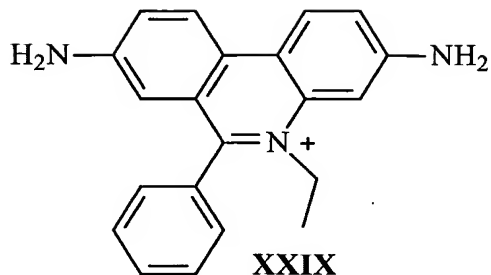
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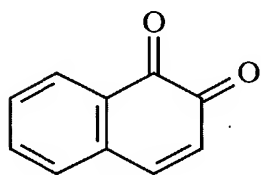
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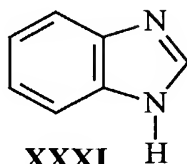
**XXVIII**  
6H-Pyrido[4,3-b]carbazole,  
5,11-dimethyl-



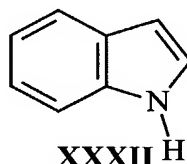
**XXIX**  
Phenanthridinium,  
3,8-diamino-5-ethyl-6-phenyl-



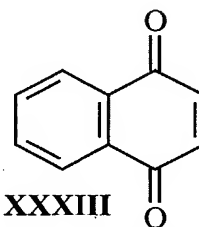
**XXX**  
1,2-Naphthalenedione



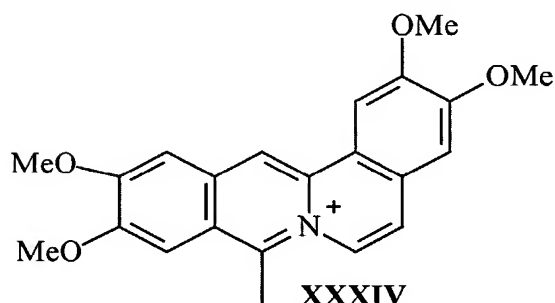
**XXXI**  
1H-Benzimidazole



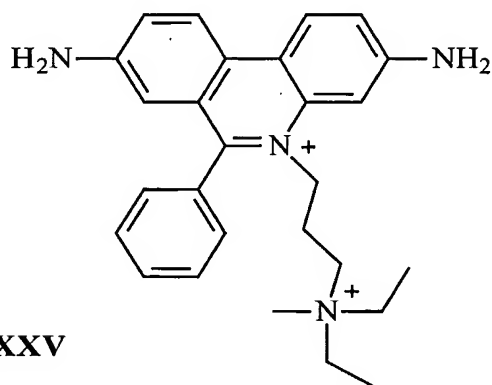
**XXXII**  
1H-Indole



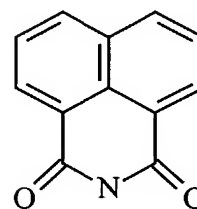
**XXXIII**  
1,4-Naphthalenedione



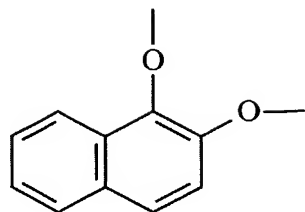
**XXXIV**  
Dibenzo[a,g]quinolizinium,  
2,3,10,11-tetramethoxy-8-methyl-



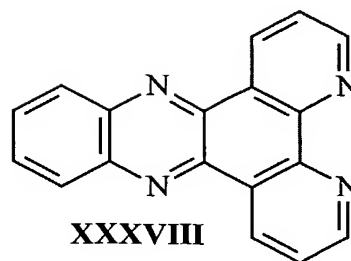
**XXXV**  
Phenanthridinium, 3,8-diamino-5-  
[3-(diethylmethylammonio)propyl]-6-phenyl-



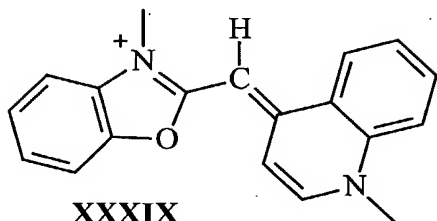
**XXXVI**  
1H-Benz[de]isoquinoline-  
1,3(2H)-dione

**XXXVII**

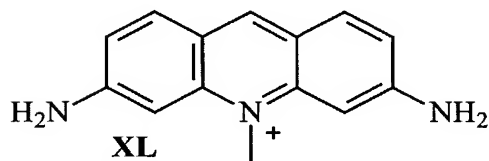
Naphthalene, 1,2-dimethoxy-

**XXXVIII**

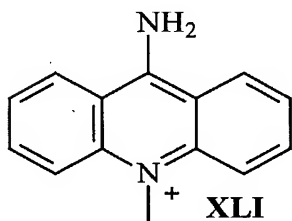
Dipyrido[3,2-a:2',3'-c]phenazine

**XXXIX**

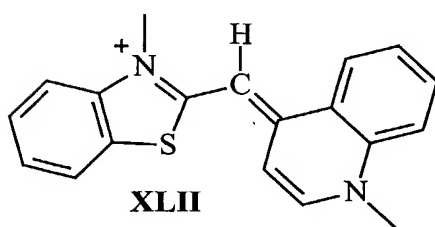
Quinolinium, 4-[(3-ethyl-2(3H)-benzoxazolylidene)methyl]-1-methyl-

**XL**

Acridine, 6-amino-3,10-dihydro-3-imino-10-methyl-

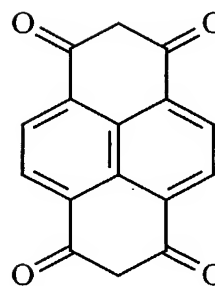
**XLI**

Acridinium, 9-amino-10-methyl-

**XLII**

Quinolinium, 1-methyl-4-

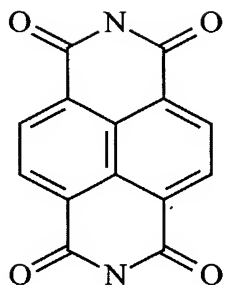
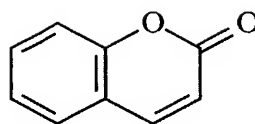
[(3-methyl-2(3H)-benzothiazolylidene)methyl]-

**XLIII**

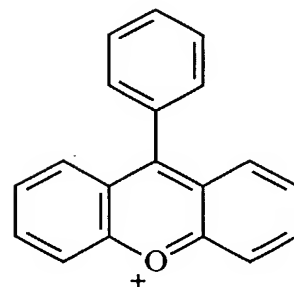
1,3,6,8(2H,7H)-Pyrenetetrone



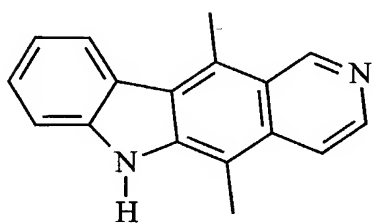
52

**XLIV**Benzo[lmn][3,8]phenanthroline-  
1,3,6,8(2H,7H)-tetrone**XLV**

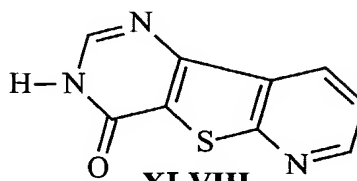
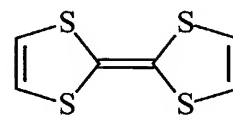
2H-1-Benzopyran-2-one

**XLVI**

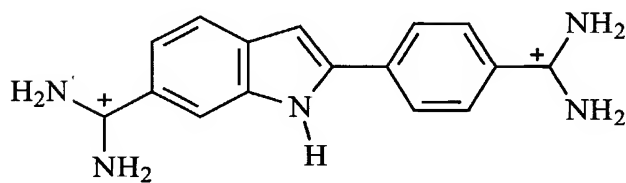
Xanthylium, 9-phenyl-

**XLVII**

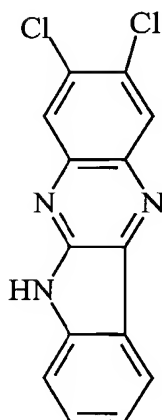
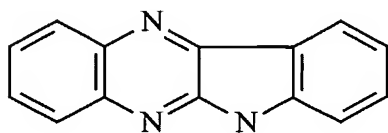
Ellipticine

**XLVIII**Pyrido[3',2':4,5]thieno  
[3,2-d]pyrimidin-  
4(1H)-one**XLIX**

Fulvalene

**L**

DAPI

**LI****LII**

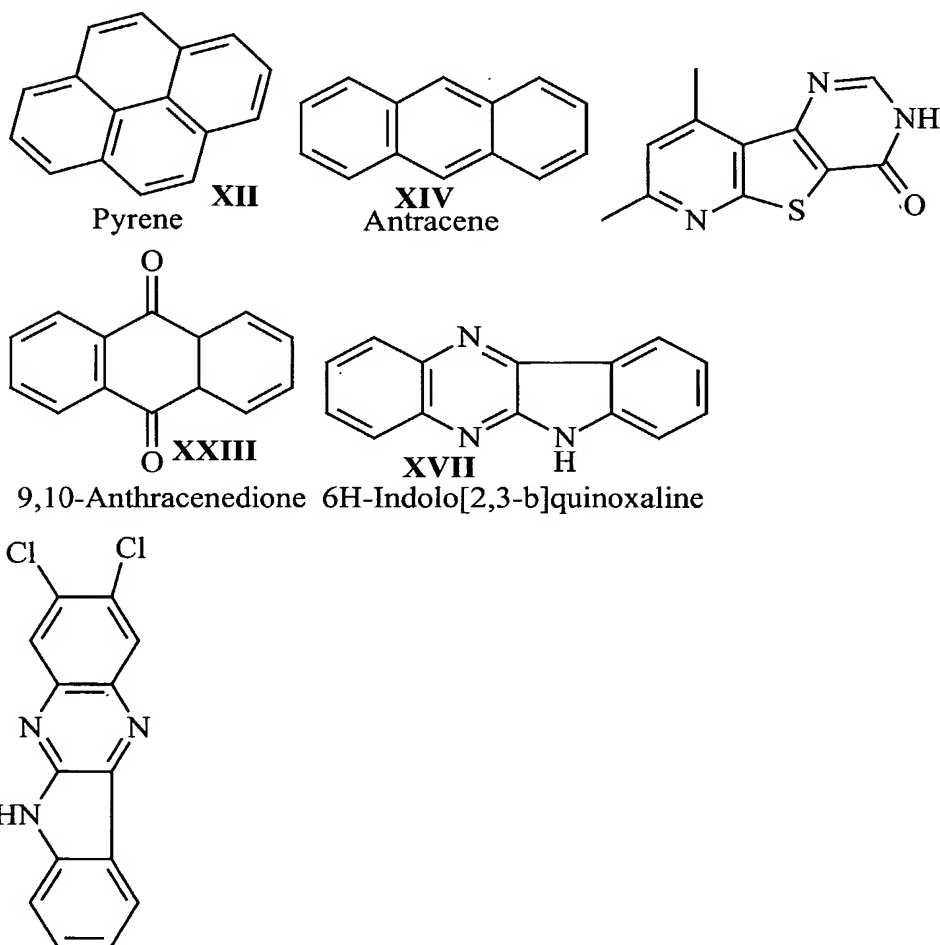
5

as well as derivatives thereof.

Even more preferably the intercalator may be selected from the group of intercalators comprising one of the intercalator structures above numbered V, XII, XIV, XV, XVII, XXIII, XXVI, XXVIII, XLVII, LI and LII as well as derivatives thereof.

5

Most preferably the intercalator is selected from the group of intercalator structures above numbered XII, XIV, XVII, XXIII, LI.



as well as derivatives thereof

15

The above list of examples is not to be understood as limiting in any way, but only as to provide examples of possible structures for use as intercalators. In addition, the substitution of one or more chemical groups on each intercalator to obtain modified structures is also included in the present invention.

5 The intercalator moiety of the intercalator pseudonucleotide is linked to the backbone unit by the linker. When going from the backbone along the linker to the intercalating moiety, the linker and intercalator connection is defined as the bond between a linker atom and the first atom being part of a conjugated system that is able to co-stack with nucleobases of a strand of a oligonucleotide or oligonucleotide analogue when said oligonucleotide or oligonucleotide analogue is hybridised to an oligonucleotide analogue comprising said intercalator pseudonucleotide.

10 In one embodiment of the present invention, the linker may comprise a conjugated system and the intercalator may comprise another conjugated system. In this case the linker conjugated system is not capable of costacking with nucleobases of said opposite oligonucleotide or oligonucleotide analogue strand.

15 Linker

The linker of a intercalator pseudonucleotide according to the present invention is a moiety connecting the intercalator and the backbone monomer of said intercalator pseudonucleotide. The linker may comprise one or more atom(s) or bond(s)  
20 between atoms.

By the definitions of backbone and intercalating moieties defined herein above, the linker is the shortest path linking the backbone and the intercalator. If the intercalator is linked directly to the backbone, the linker is a bond.

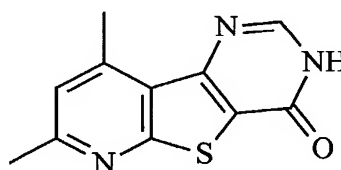
25 The linker usually consists of a chain of atoms or a branched chain of atoms. Chains can be saturated as well as unsaturated. The linker may also be a ring structure with or without conjugated bonds.

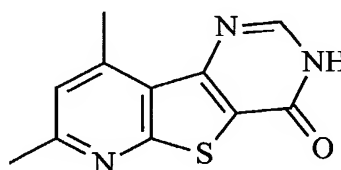
30 For example the linker may comprise a chain of m atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit.

In some embodiments the total length of the linker and the intercalator of the intercalator pseudonucleotides according to the present invention preferably is between 8 and 13 Å (see herein below). Accordingly, m should be selected dependent on the size of the intercalator of the specific intercalator pseudonucleotide.

I.e. m should be relatively large, when the intercalator is small and m should be relatively small when the intercalator is large. For most purposes however m will be an integer from 1 to 7, such as from 1-6, such as from 1-5, such as from 1-4. As described above the linker may be an unsaturated chain or another system involving conjugated bonds. For example the linker may comprise cyclic conjugated structures. Preferably, m is from 1 to 4 when the linker is an saturated chain.

When the intercalator is pyrene, m is preferably an integer from 1 to 7, such as from 1-6, such as from 1-5, such as from 1-4, more preferably from 1 to 4, even more preferably from 1 to 3, most preferably m is 2 or 3.



When the intercalator has the structure , m is preferably from 2 to 6, more preferably 2.

The chain of the linker may be substituted with one or more atoms selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

In one embodiment the linker is an azaalkyl, oxaalkyl, thiaalkyl or alkyl chain. For example the linker may be an alkyl chain substituted with one or more selected from the group consisting C, H, O, S, N, P, Se, Si, Ge, Sn and Pb. In a preferred embodiment the linker consists of an unbranched alkyl chain, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit and wherein each C is substituted with 2 H. More preferably, said unbranched alkyl chain is from 1 to 5 atoms long, such as from 1 to 4 atoms long, such as from 1 to 3 atoms long, such as from 2 to 3 atoms long.

In another embodiment of the invention the linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb. For example the linker may be such a ring structure substituted with one or more  
5 selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

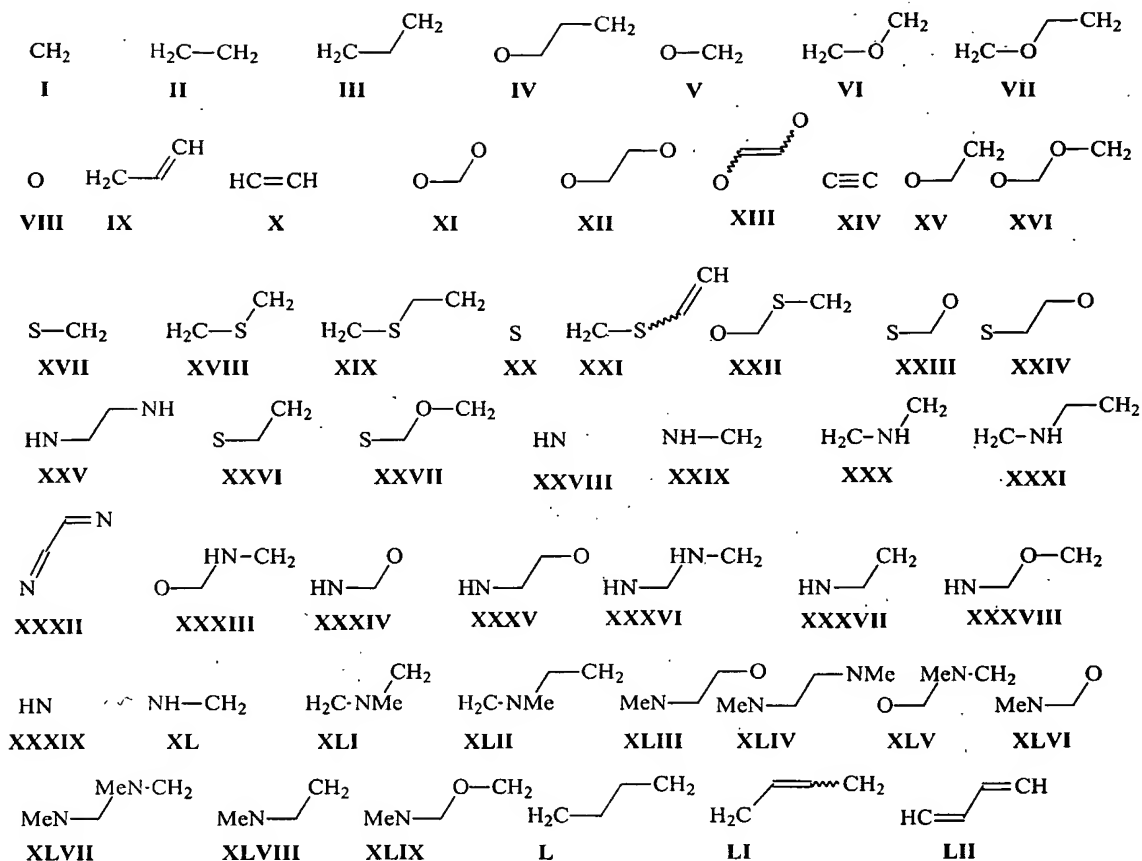
In another embodiment the linker consists of from 1-6 C atoms, from 0-3 of each of the following atoms O, S, N. More preferably the linker consists of from 1-6 C atoms and from 0-1 of each of the atoms O, S, N.  
10

In a preferred embodiment the linker consists of a chain of C, O, S and N atoms, optionally substituted. Preferably said chain should consist of at the most 3 atoms, thus comprising from 0 to 3 atoms selected individually from C, O, S, N, optionally substituted.  
15

In a preferred embodiment the linker consists of a chain of C, N, S and O atoms, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit.

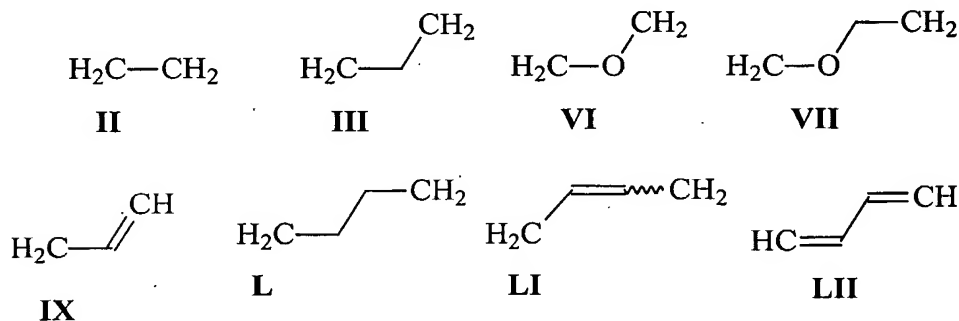
20 Preferably such a chain comprise one of the linkers shown below, most preferably the linker consist of one of the molecule shown below:

57

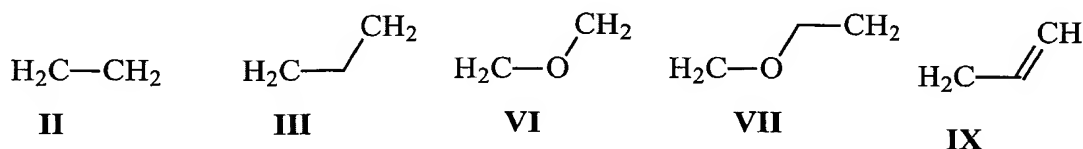


In a preferred embodiment the chain comprise one of the linkers shown below, more preferably the linker consist of one of the molecule shown below:

5



In a more preferred embodiment the chain comprise one of th linkers shown below, more preferably the linker consist of one of the molecule shown below:



5

The linker constitutes Y in the formula for the intercalator pseudonucleotide X-Y-Q, as defined above, and hence X and Q are not part of the linker.

#### Intercalator pseudonucleotides

10 Intercalator pseudonucleotides according to the present invention preferably have the general structure



15 wherein

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue,

20 Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

25

wherein the total length of Q and Y is in the range from 7 Å to 20 Å,

with the proviso that when the intercalator is pyrene the total length of Q and Y is in the range from 9 Å to 13 Å.

30

Furthermore, in a preferred embodiment of the present invention the intercalator pseudonucleotide comprises a backbone monomer unit, wherein said backbone

monomer unit is capable of being incorporated into the phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 4 atoms are separating the two phosphor atoms of the backbone that are closest to the intercalator.

5

The intercalator pseudonucleotides preferably do not comprise a nucleobase capable of forming Watson-Crick hydrogen bonding. Hence intercalator pseudonucleotides according to the invention are preferably not capable of Watson-Crick base pairing.

10

Preferably, the total length of Q and Y is in the range from 7 Å to 20 Å, more preferably, from 8 Å to 15 Å, even more preferably from 8 Å to 13 Å, even more preferably from 8.4 Å to 12 Å, most preferably from 8.59 Å to 10 Å or from 8.4 Å to 10.5 Å.

15

When the intercalator is pyrene the total length of Q and Y is preferably in the range of 8 Å to 13 Å, such as from 9 Å to 13 Å, more preferably from 9.05 Å to 11 Å, such as from 9.0 Å to 11 Å, even more preferably from 9.05 to 10 Å, such as from 9.0 to 10 Å, most preferably about 9.8 Å.

20

The total length of the linker (Y) and the intercalator (Q) should be determined by determining the distance from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest away from the backbone monomer unit. Preferably, the distance should be the maximal distance in which bonding angles and normal chemical laws are not broken or distorted in any way.

25

The distance should preferably be determined by calculating the structure of the free intercalating pseudonucleotide with the lowest conformational energy level, and then determining the maximum distance that is possible from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest away from the backbone monomer unit without bending, stretching or otherwise distorting the structure more than simple rotation of bonds

35



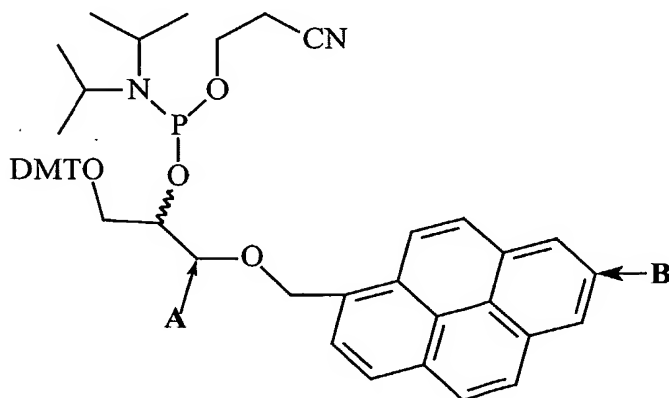
that are free to rotate (e.g. not double bonds or bonds participating in a ring structure).

Preferably the energetically favorable structure is found by *ab initio* or forcefields calculations.

Even more preferably the distance should be determined by a method consisting of the following steps:

- a) the structure of the intercalator pseudonucleotide of interest is drawn by computer using the programme ChemWindow® 6.0 (BioRad); and
- b) the structure is transferred to the computer programme SymApps™ (BioRad); and
- c) the 3-dimensional structure comprising calculated lengths of bonds and bonding angles of the intercalator pseudonucleotide is calculated using the computer programme SymApps™ (BioRad); and
- d) the 3 dimensional structure is transferred to the computer programme RasWin Molecular Graphics Ver. 2.6-ucb; and
- e) the bonds are rotated using RasWin Molecular Graphics Ver. 2.6-ucb to obtain the maximal distance (the distance as defined herein above); and
- f) the distance is determined.

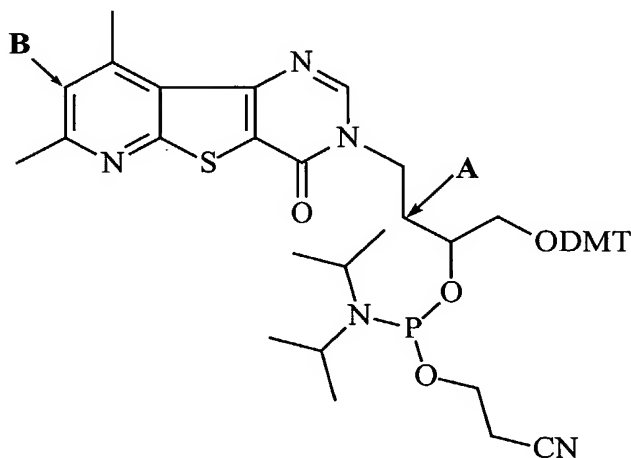
For example when the intercalator pseudonucleotide has the following structure:



61

the total length of Q and Y is determined by measuring the linear distance from the center of the atom at A to the center of the atom at B, which in the above example is 9,79 Å.

- 5 In another example the intercalator pseudonucleotide has the following structure:

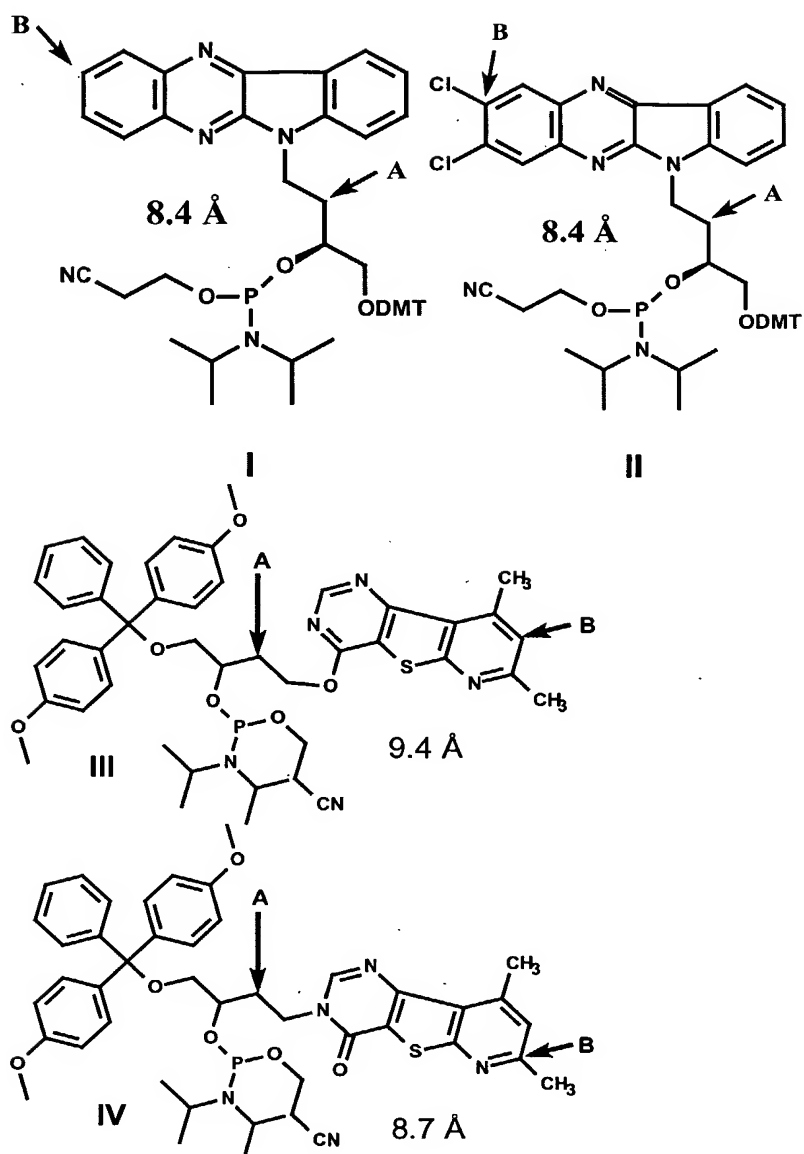


- 10 The total length of Q and Y, which is measured in a straight line from the center of the atom at A to the center of the atom at B is 8.71 Å.

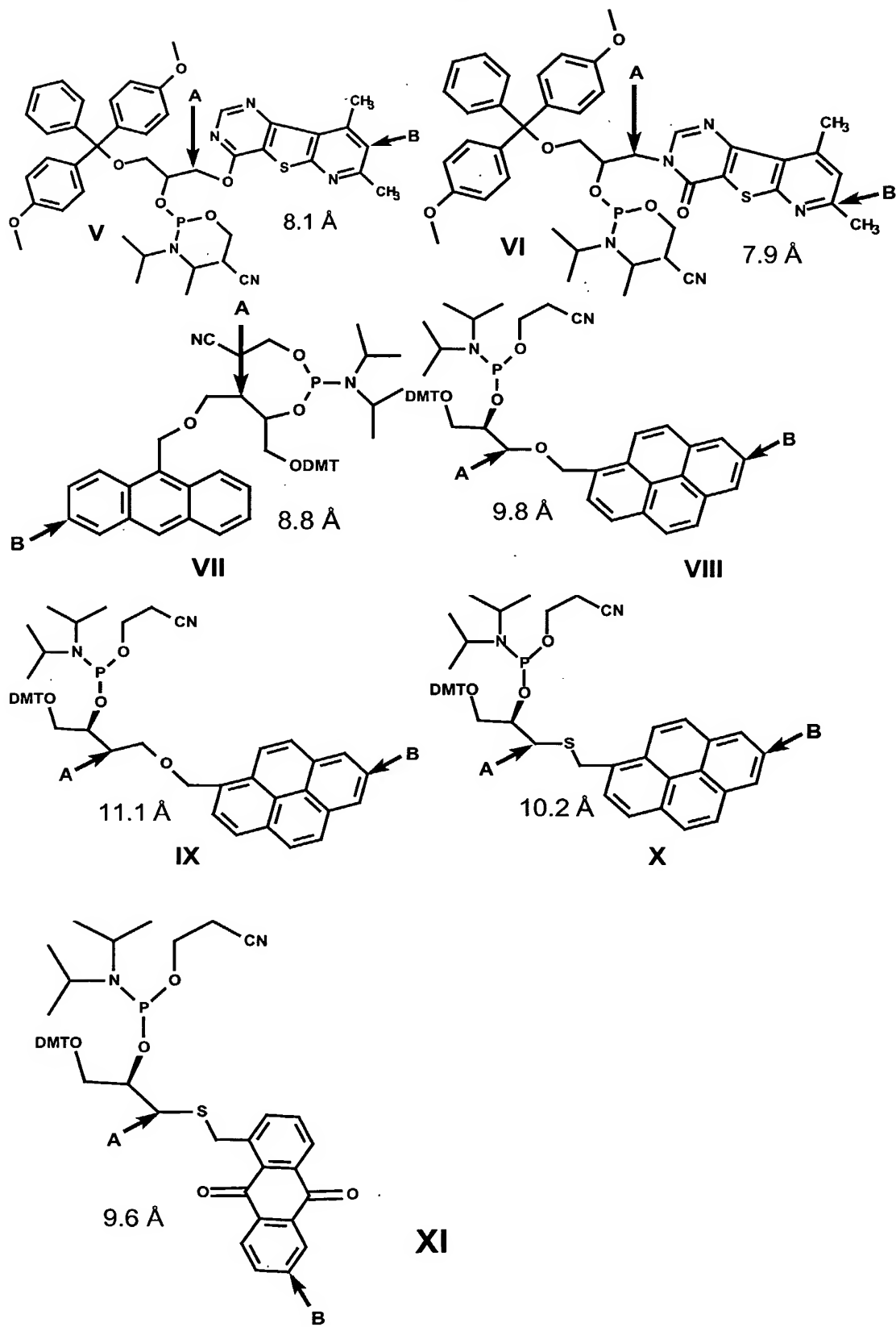
Below here a measure for the length measured in a straight line for a preferred series of intercalator pseudonucleotides is disclosed:

15

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63

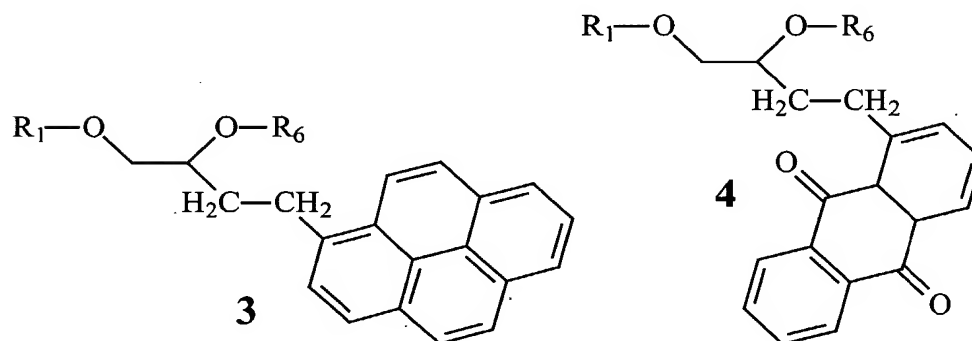
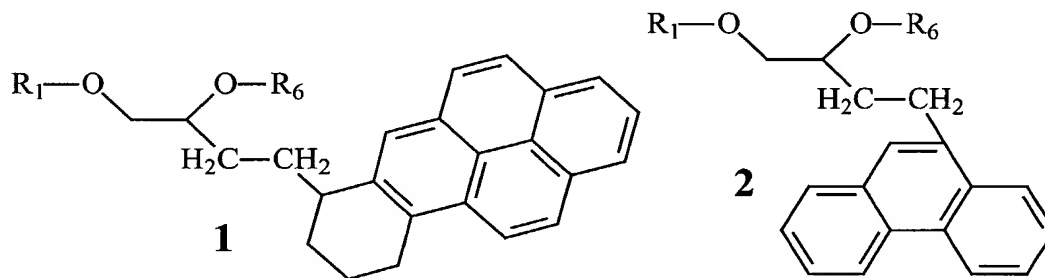


Intercalator pseudonucleotides according to the present invention may be any combination of the above mentioned backbone monomer units, linkers and intercalators.

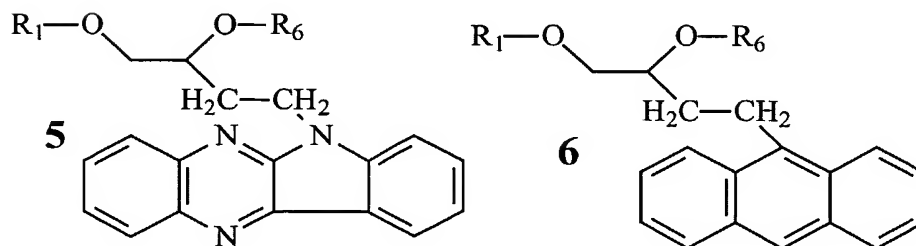
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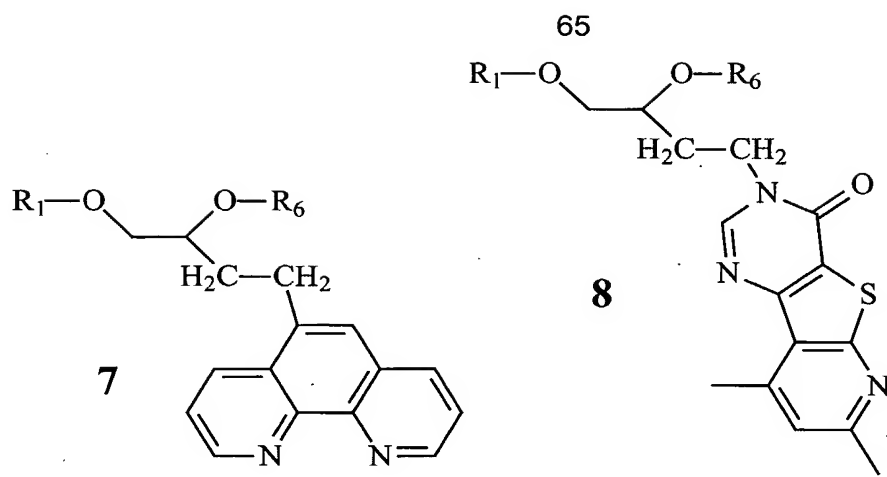
In one embodiment of the invention the intercalator pseudonucleotide is selected from the group consisting of intercalator pseudonucleotides with the structures 1) to 9 as indicated herein below:

10

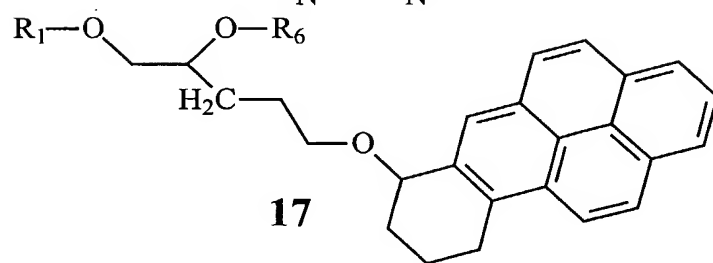
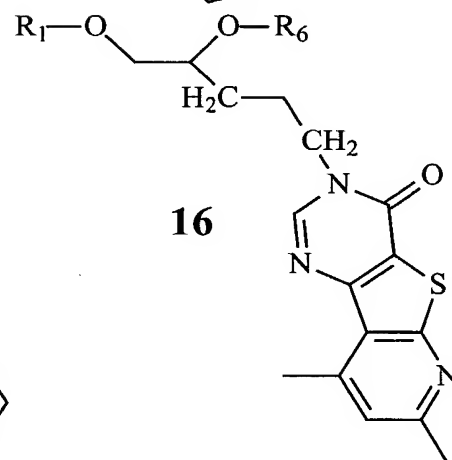
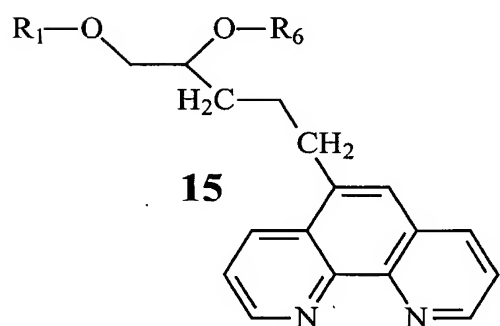
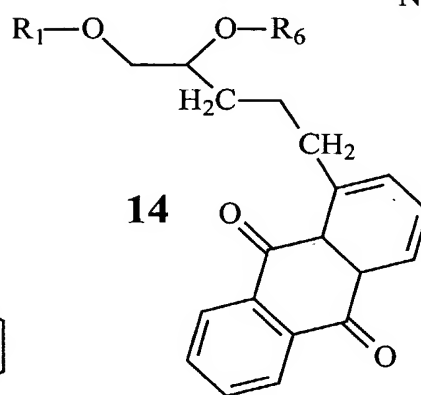
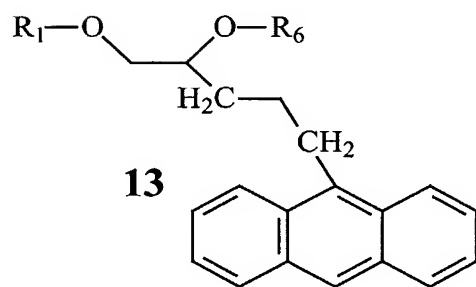
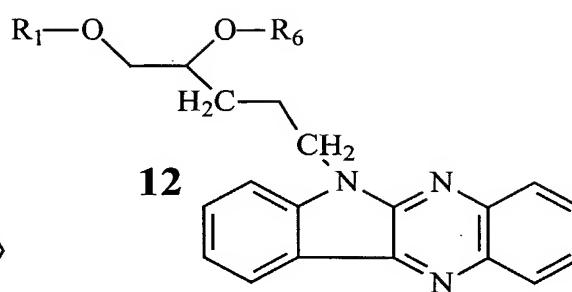
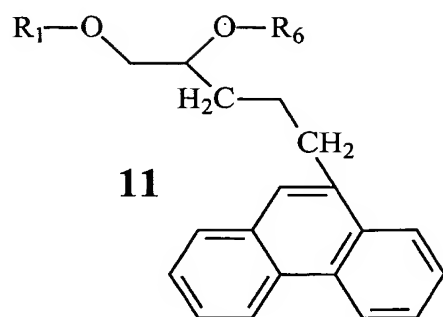


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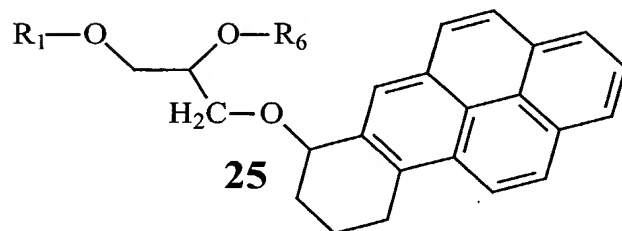
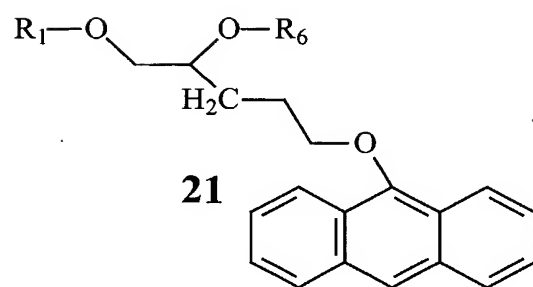
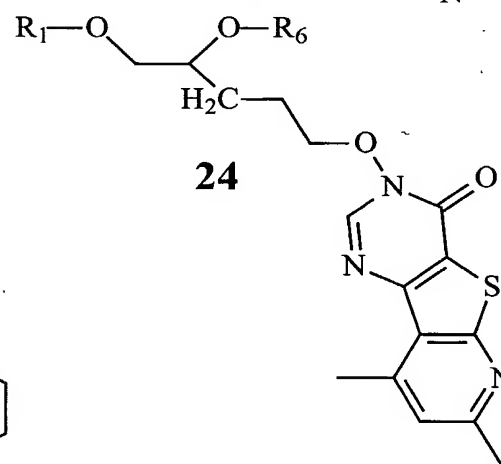
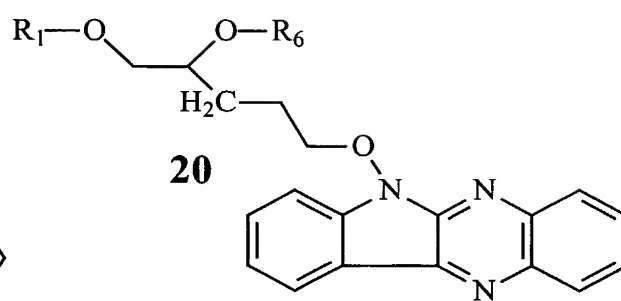
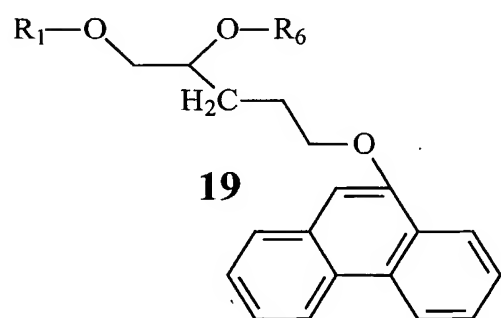
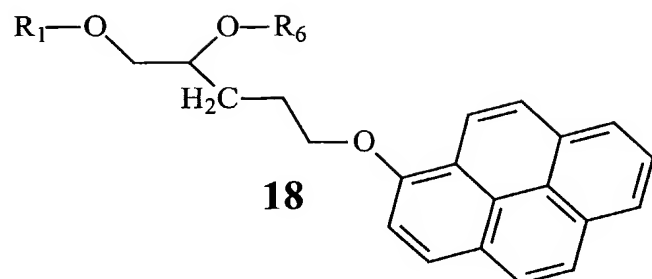




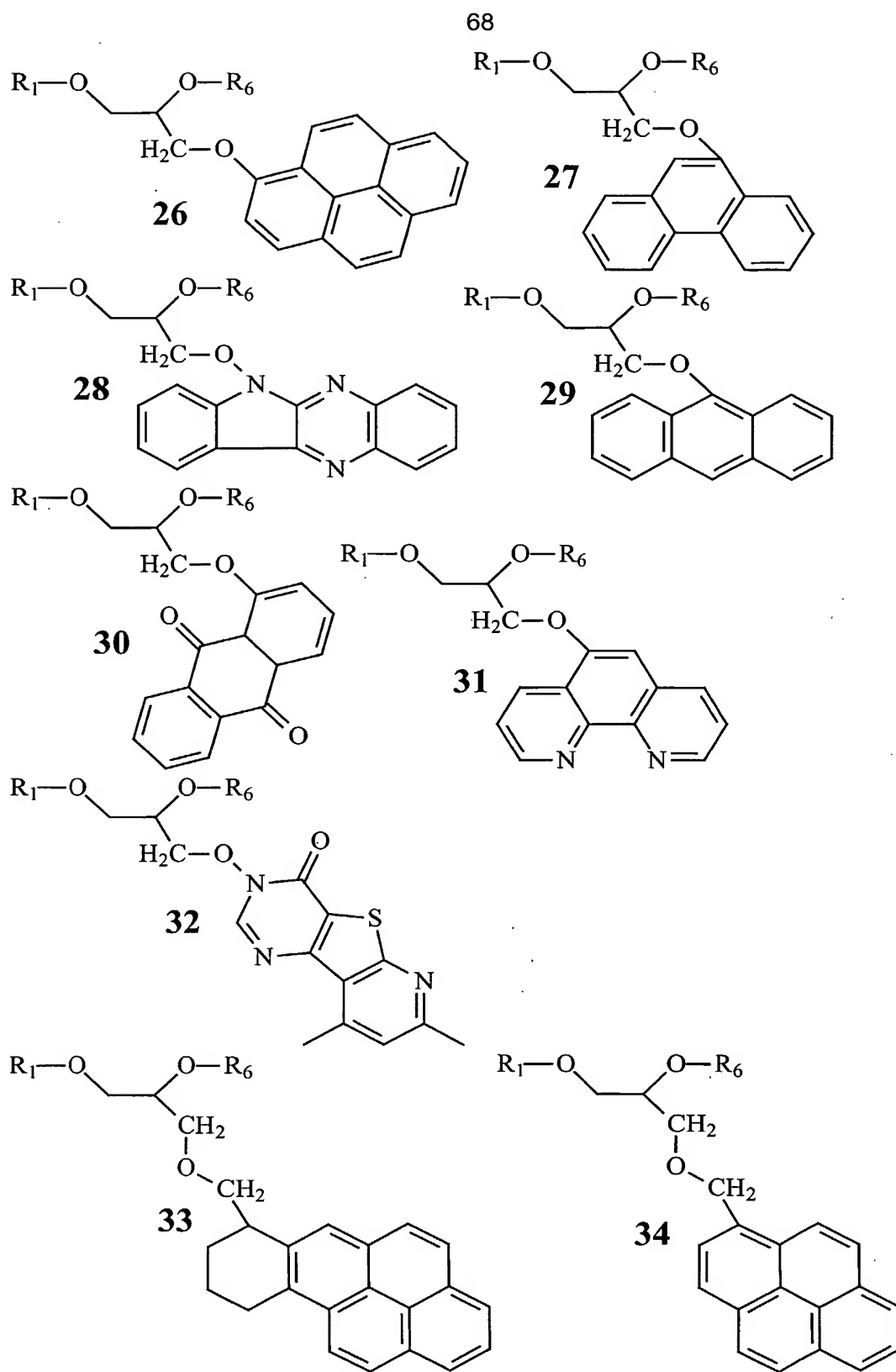
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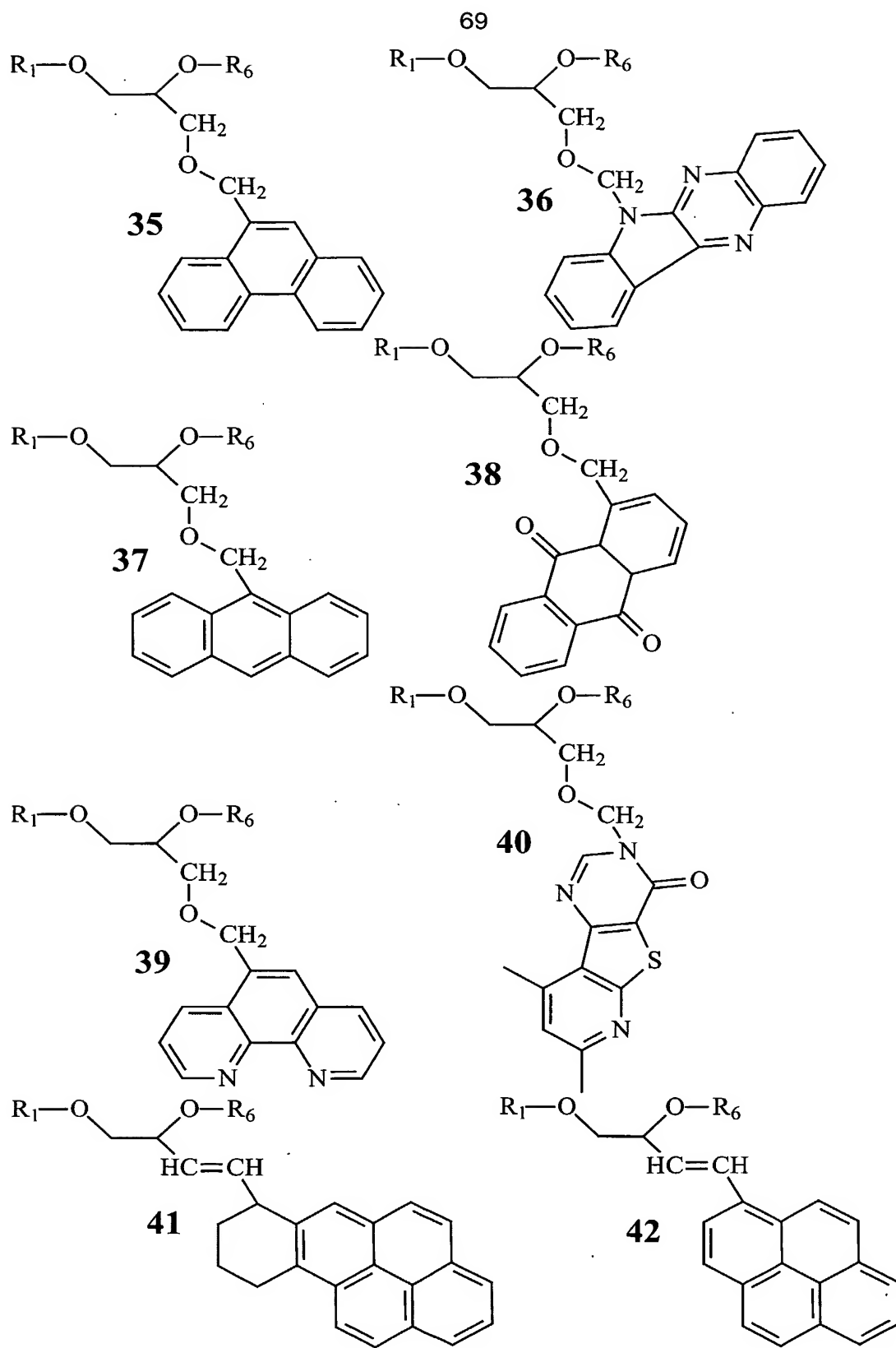


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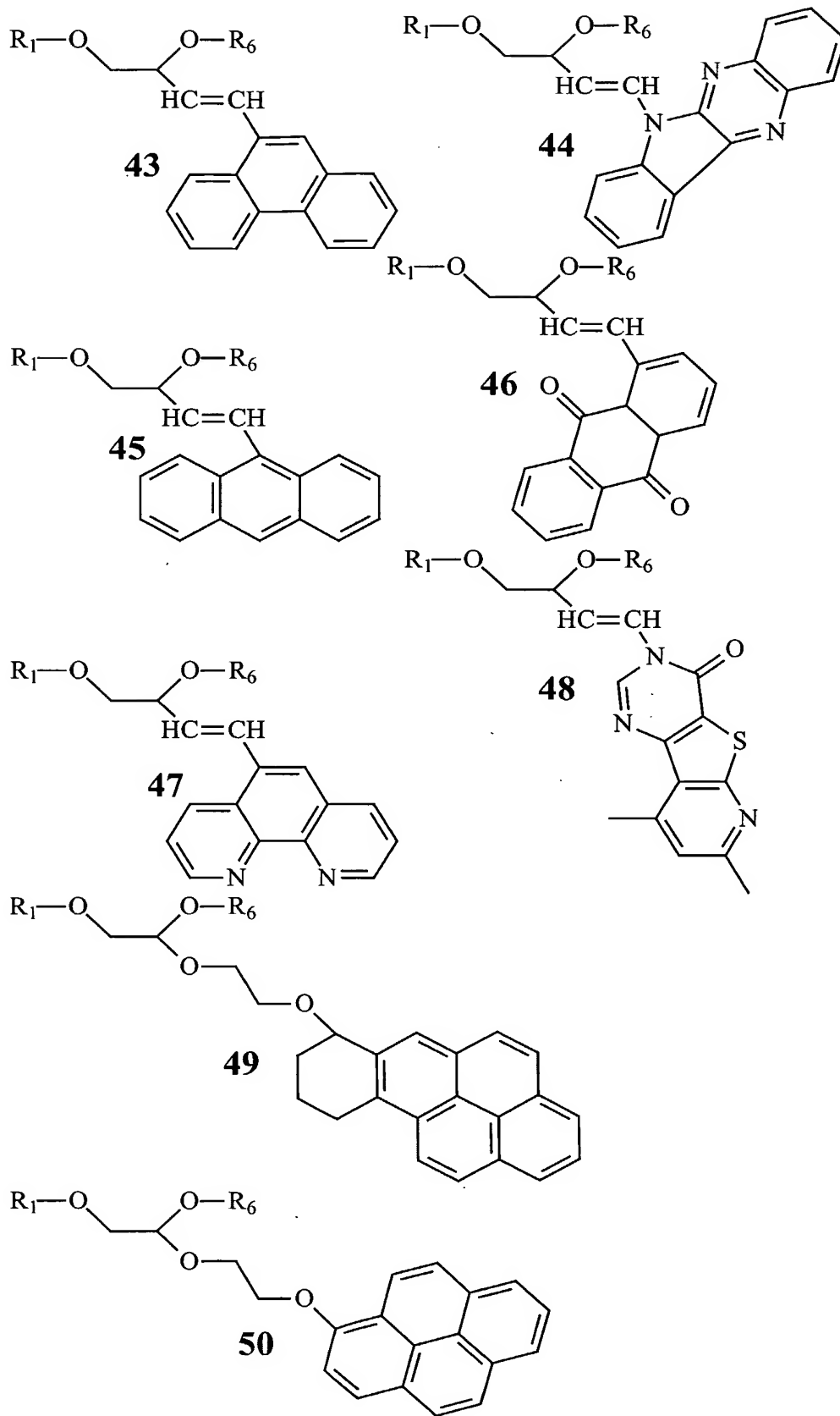




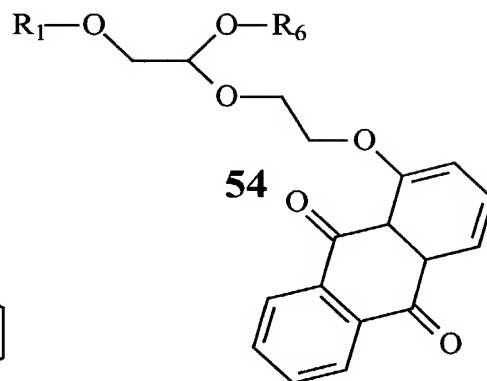
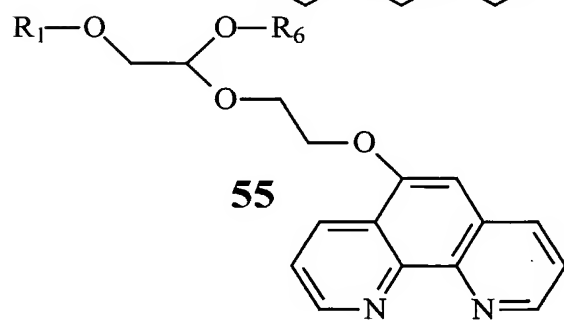
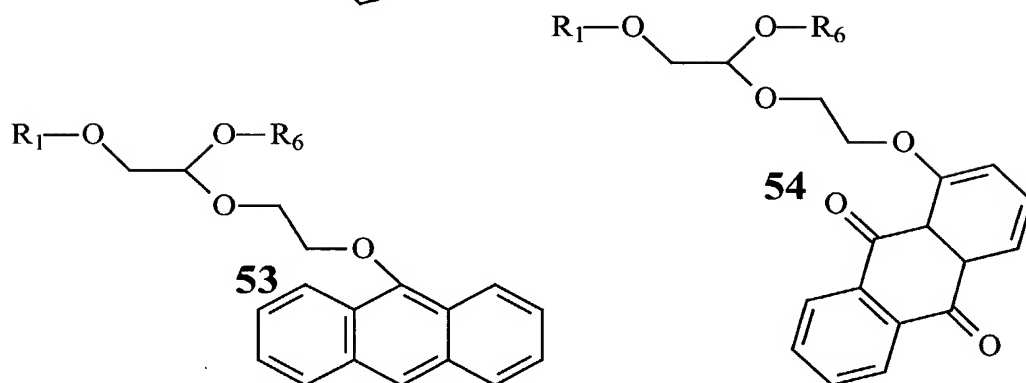
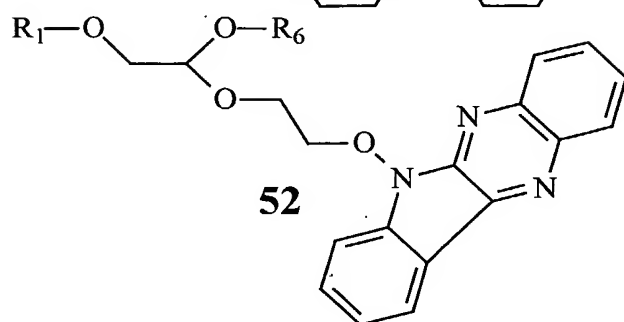
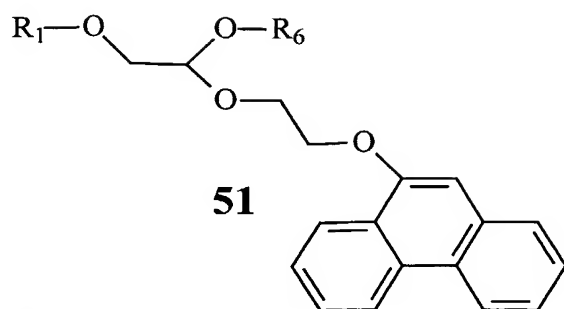




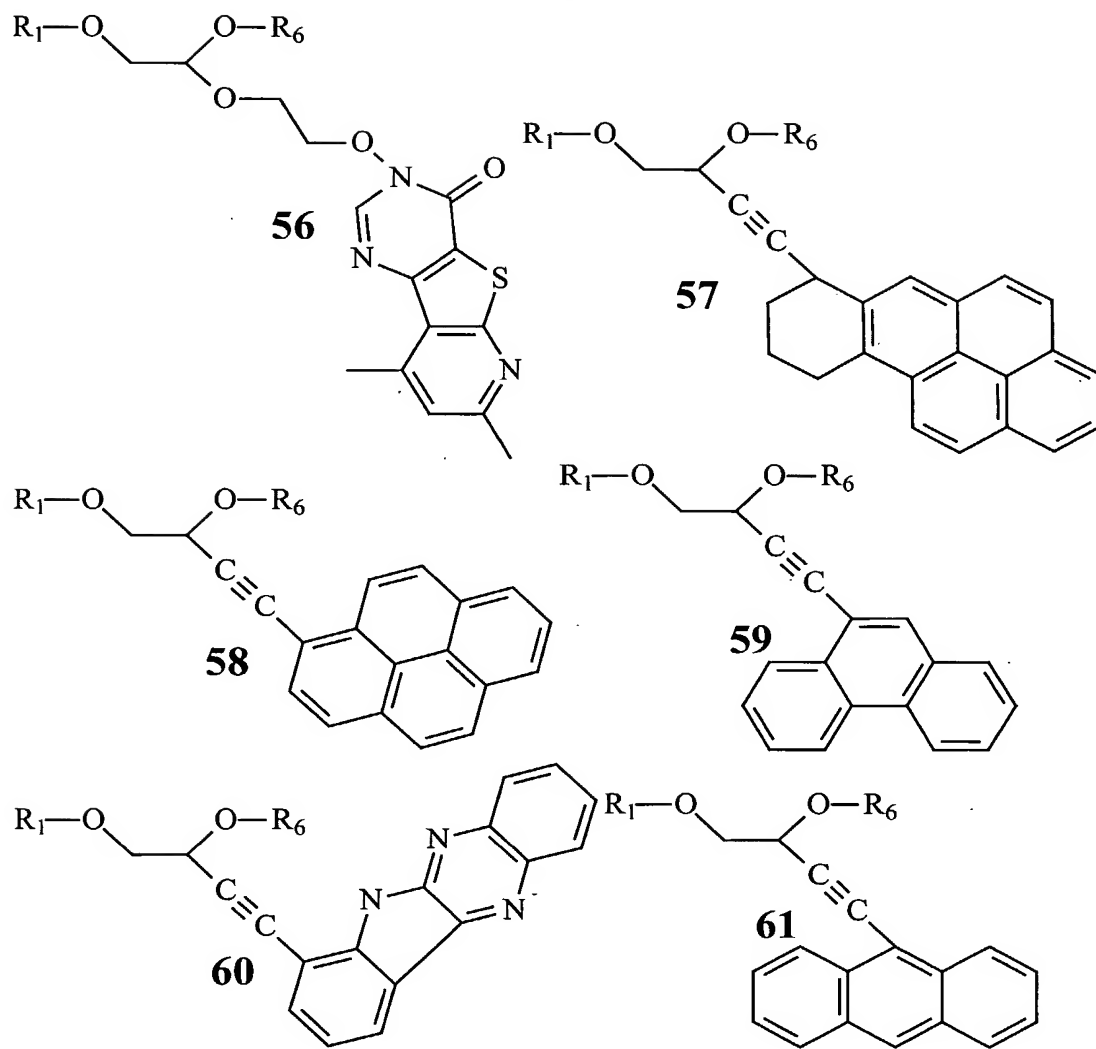
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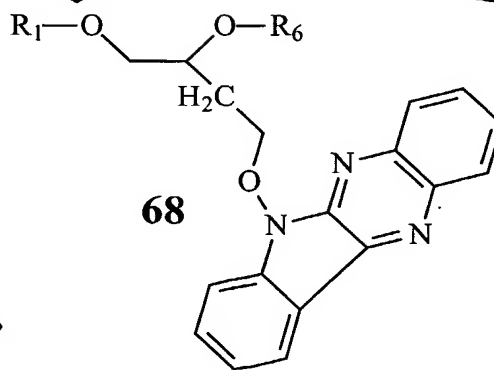
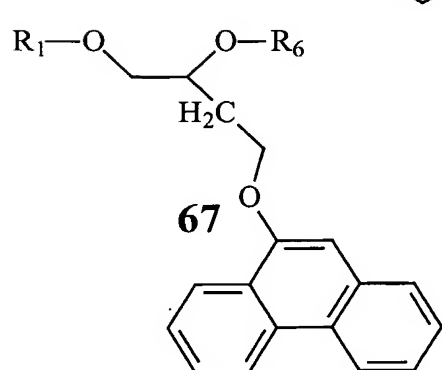
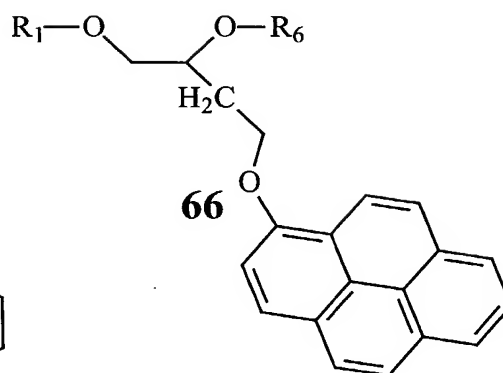
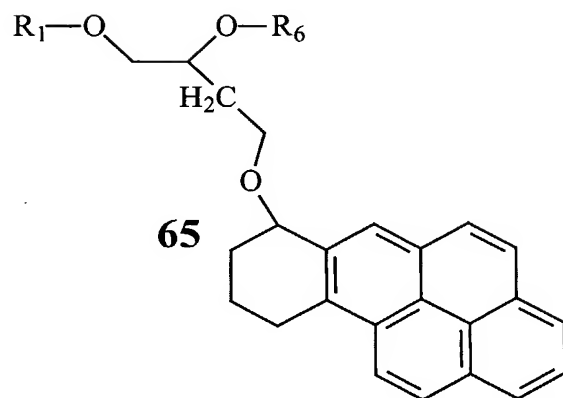
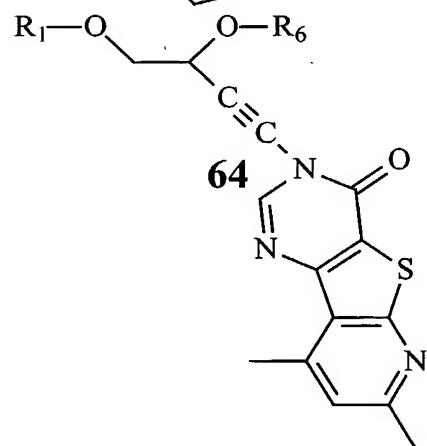
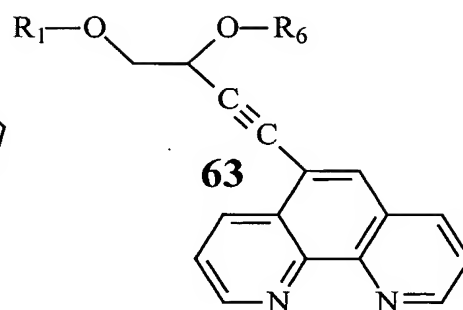
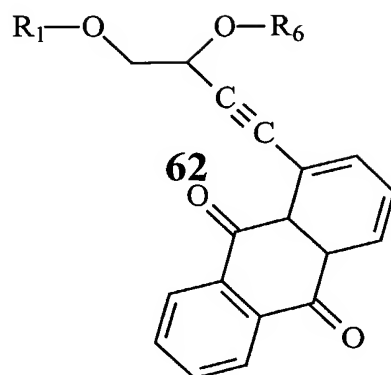
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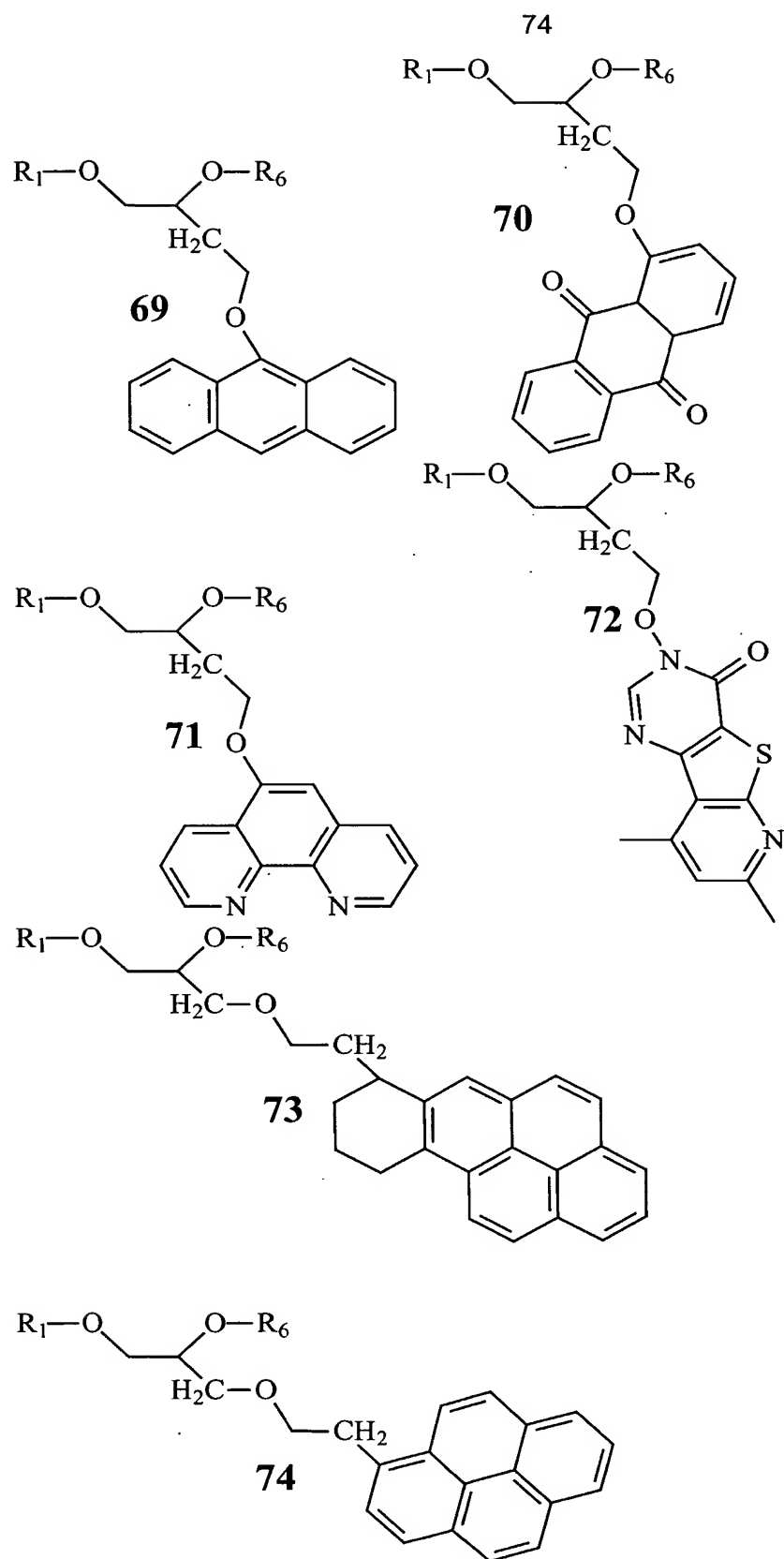


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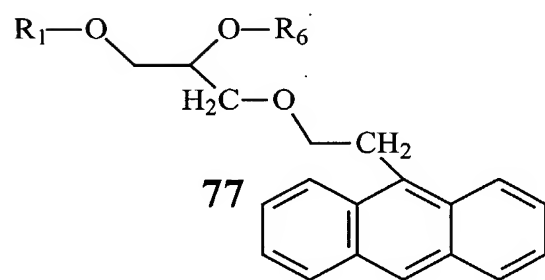
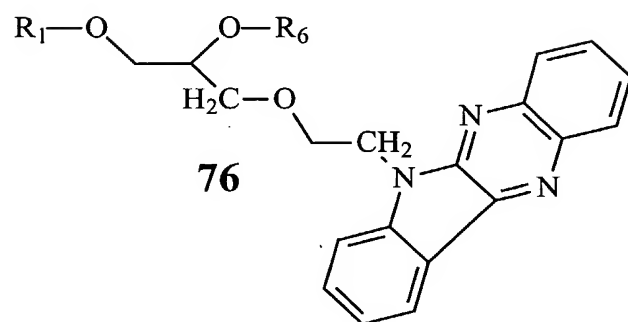
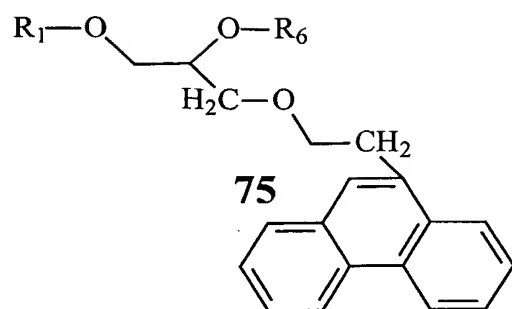


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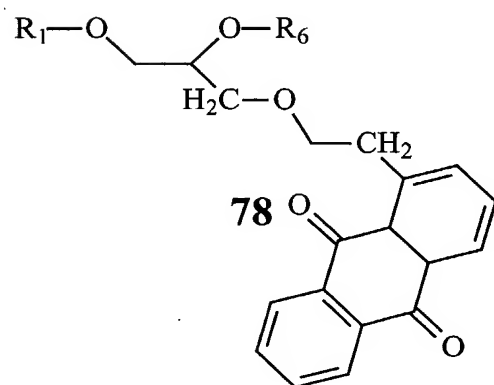




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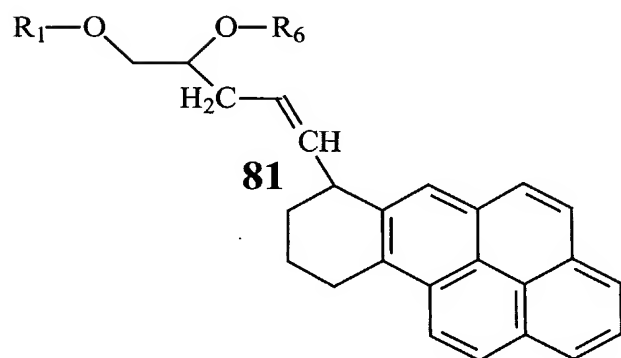
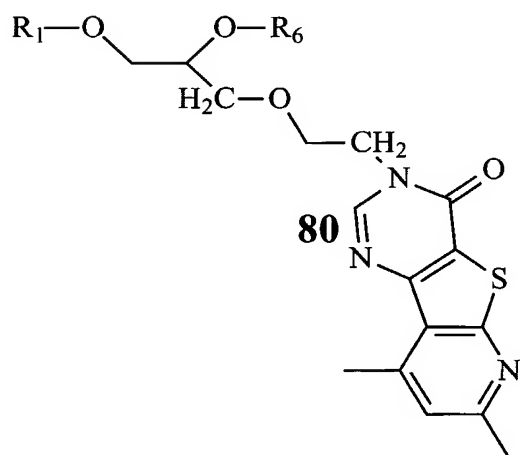
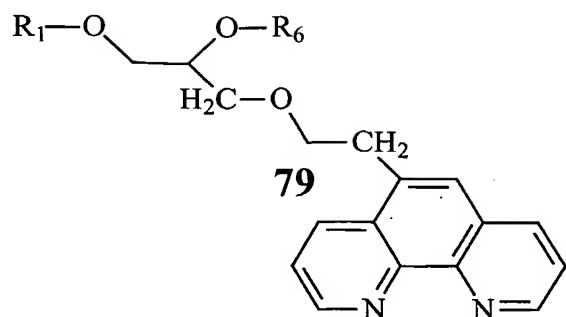


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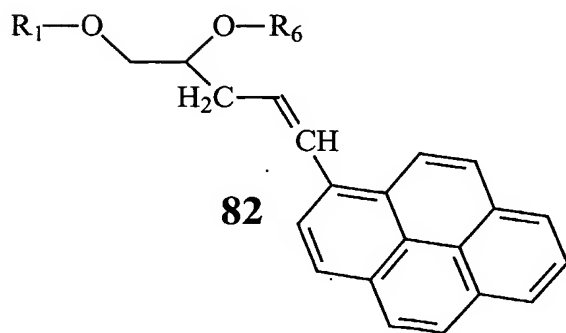




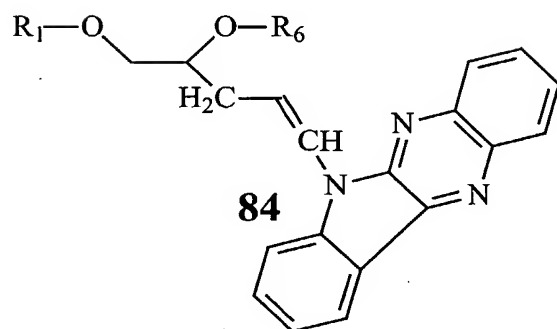
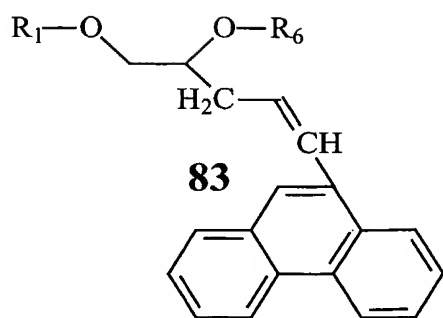
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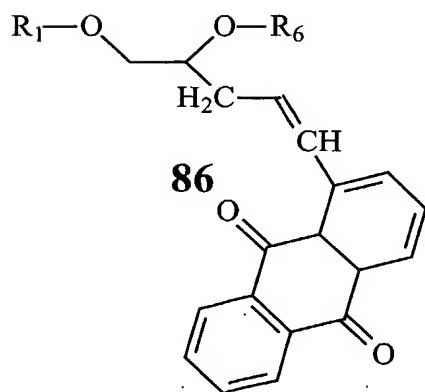
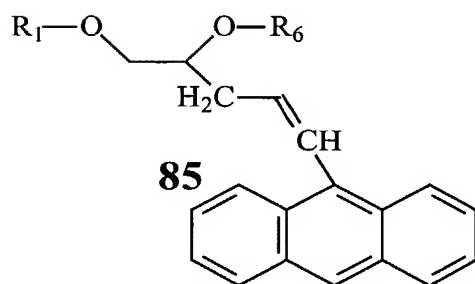
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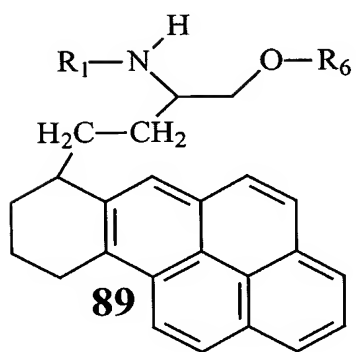
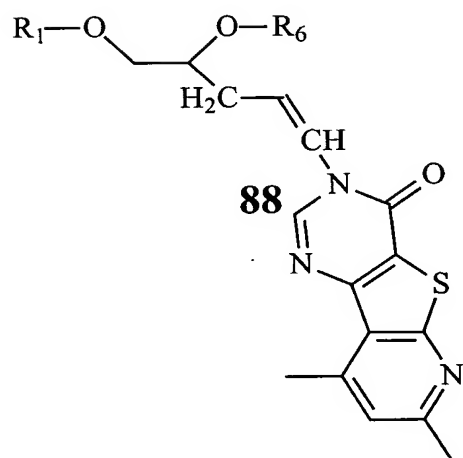
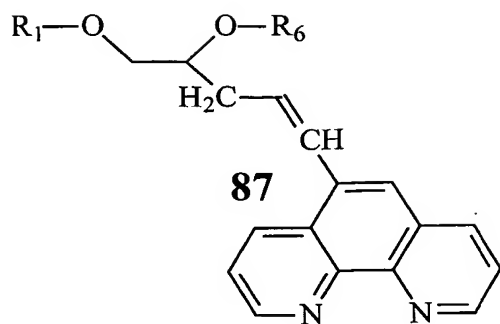


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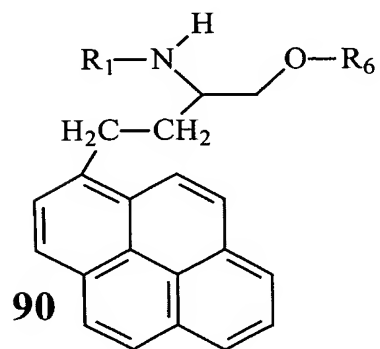


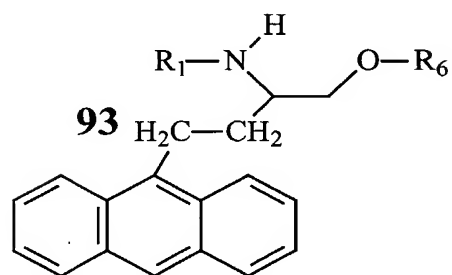
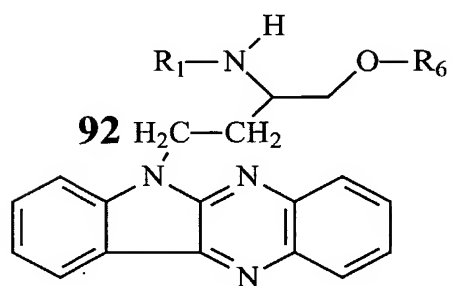
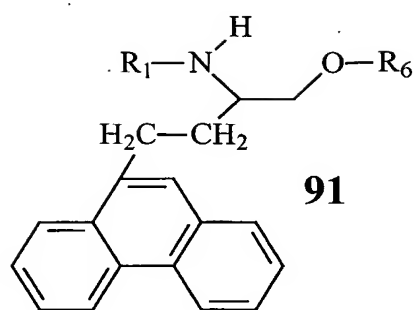
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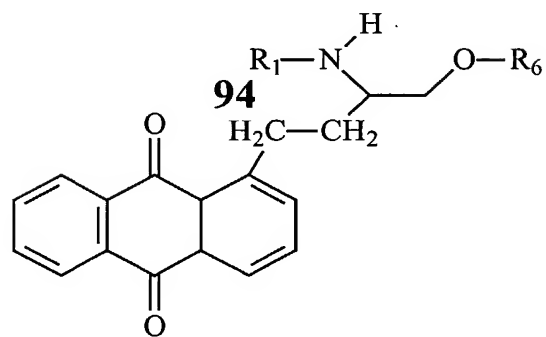


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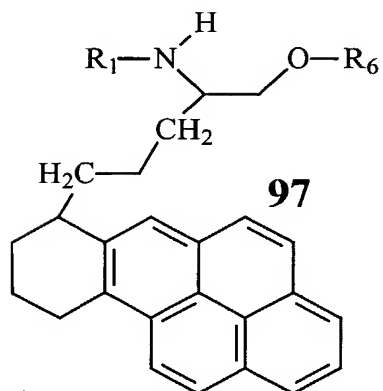
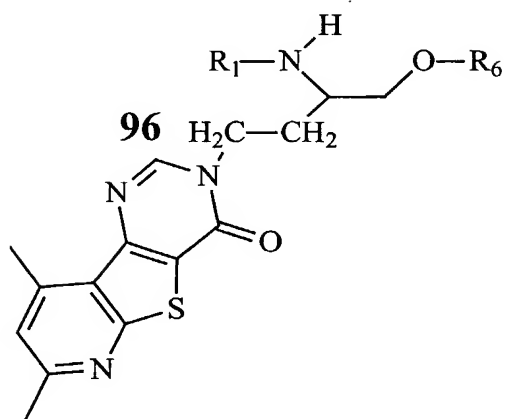
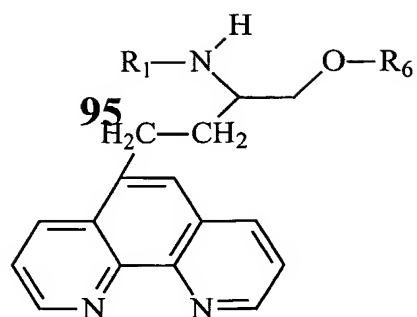


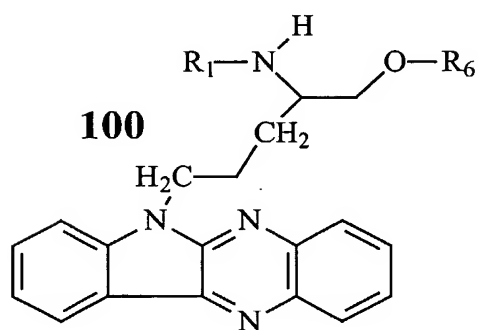
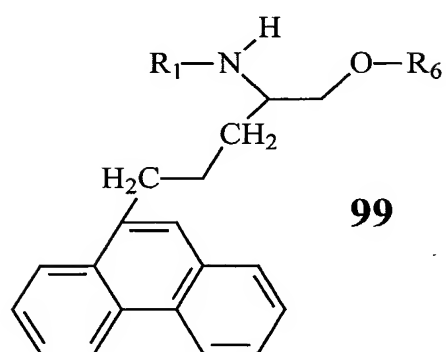
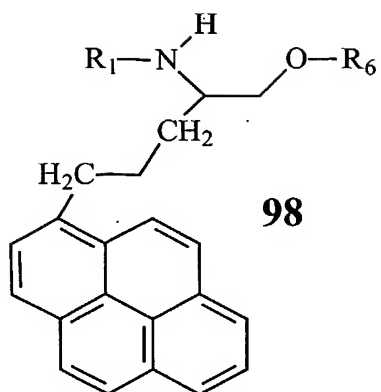


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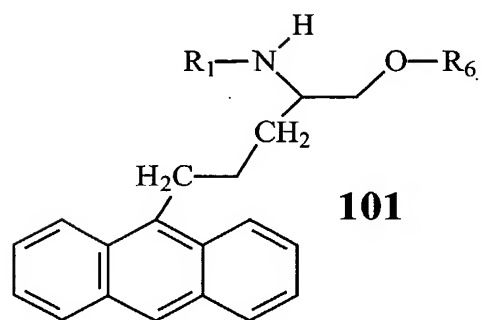


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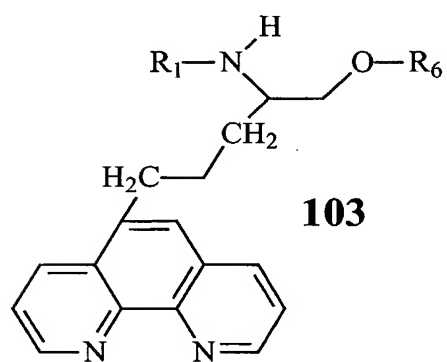
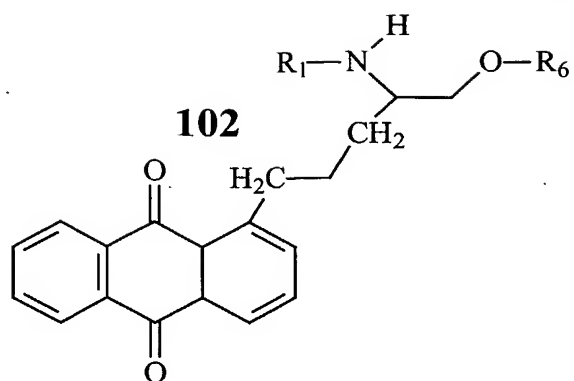


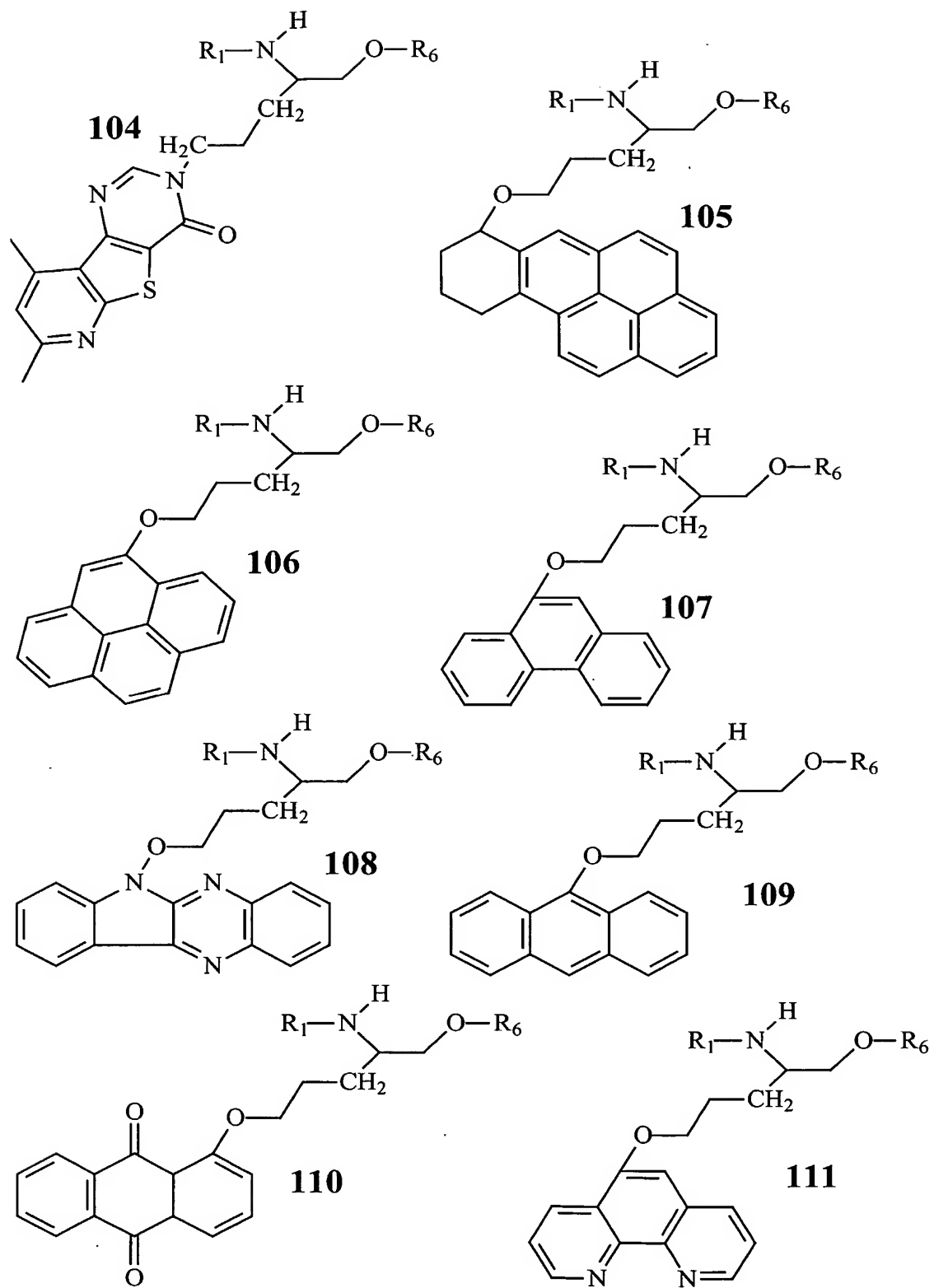


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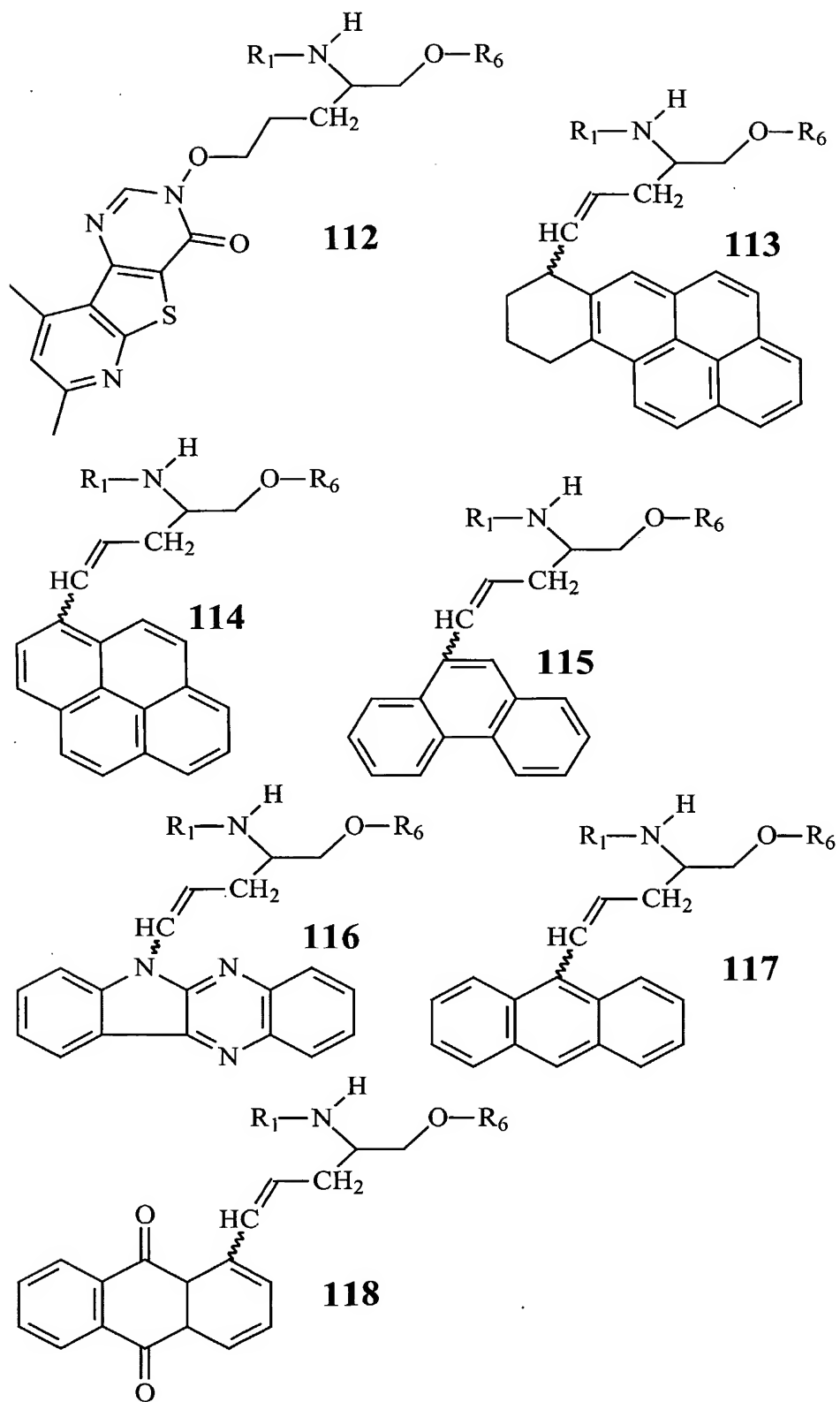


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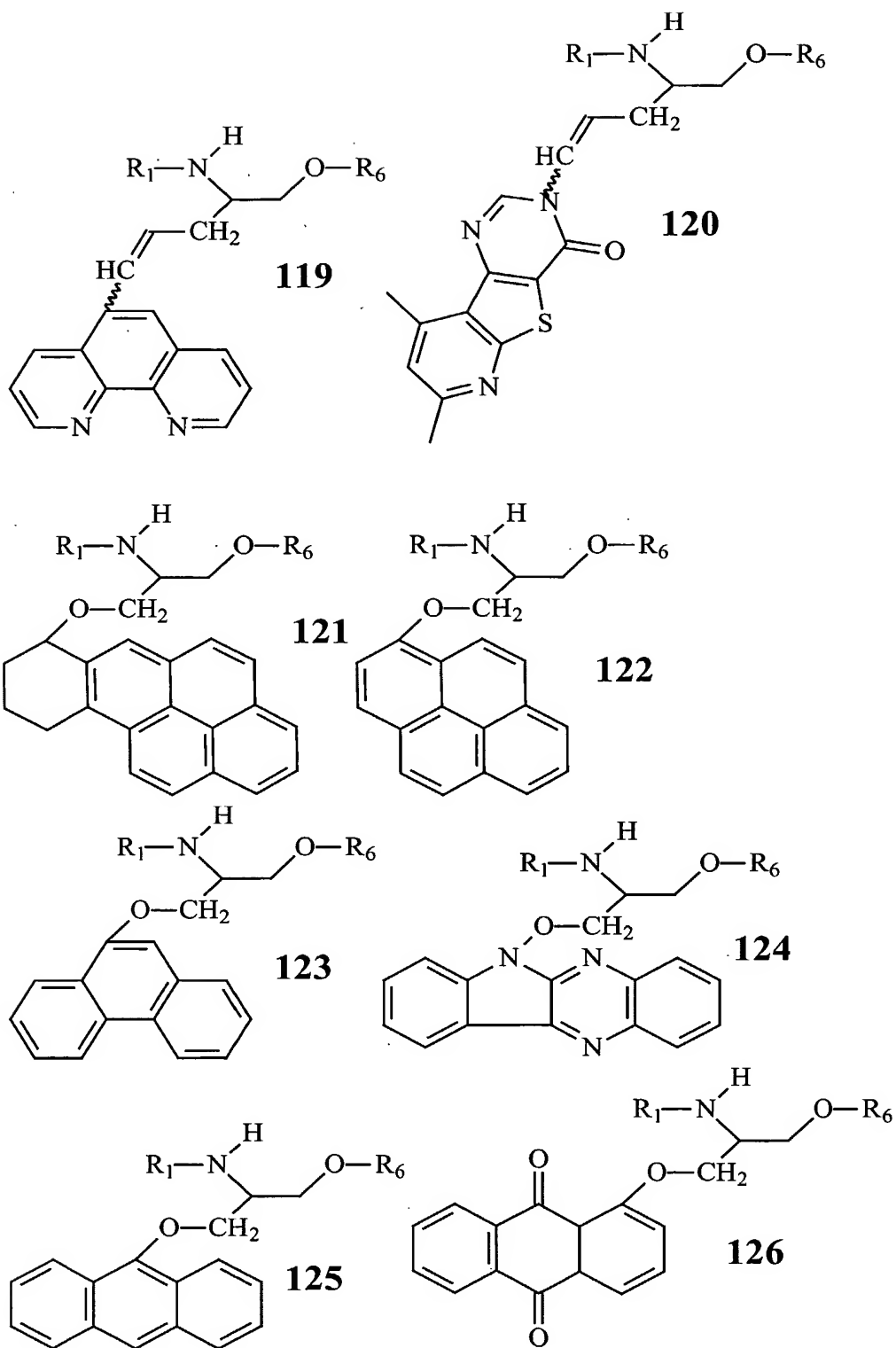




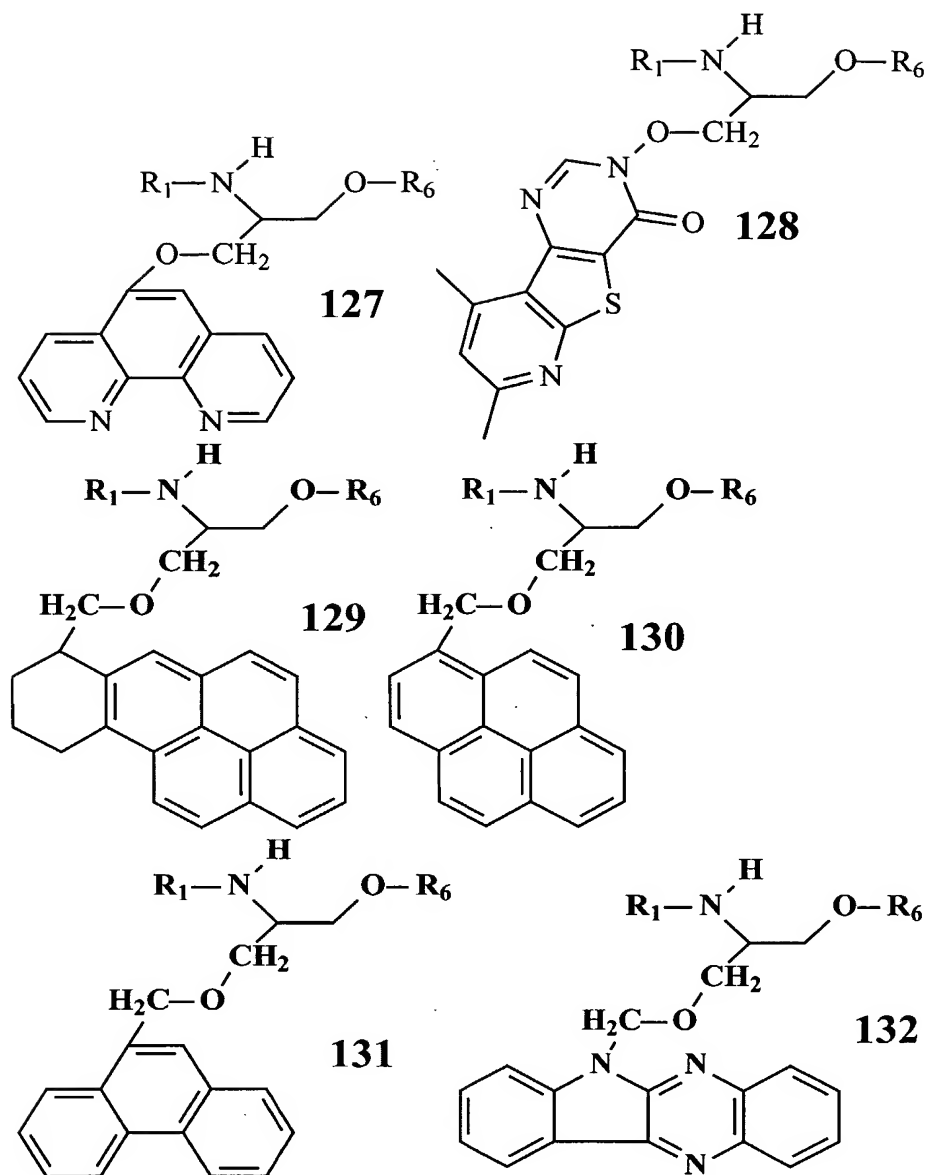


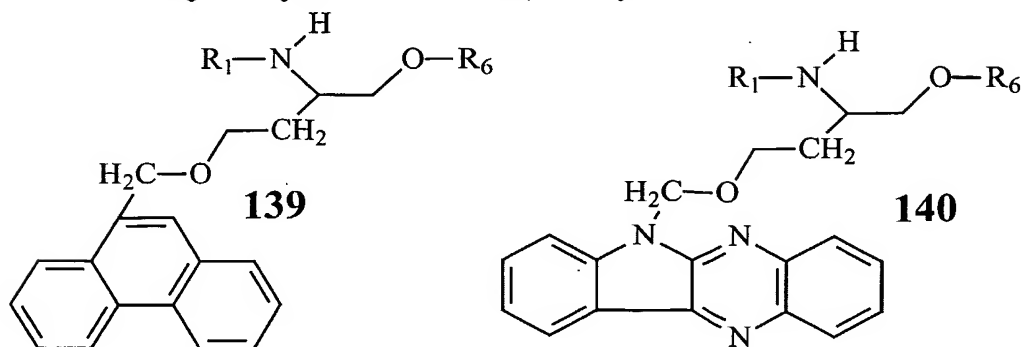
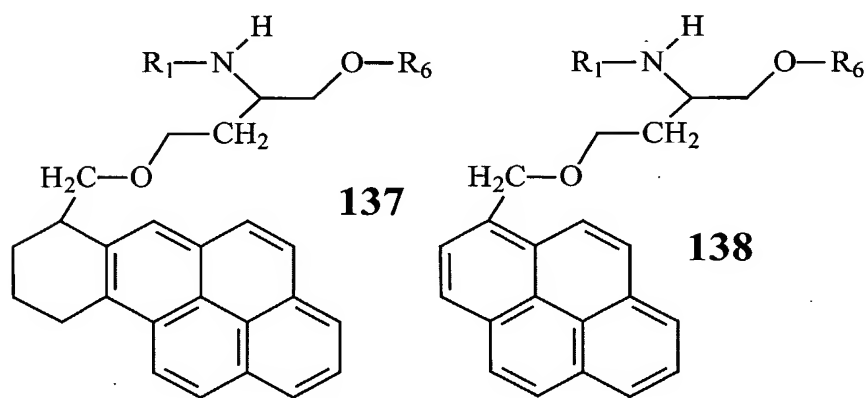
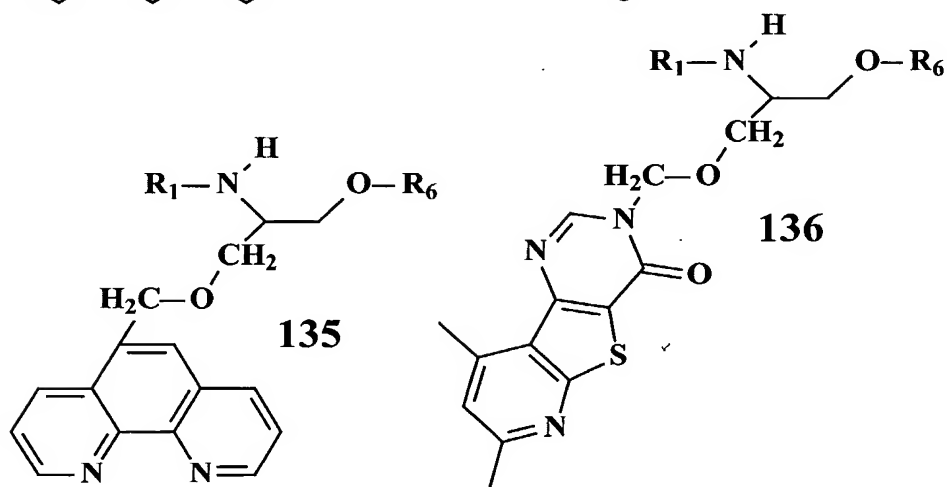
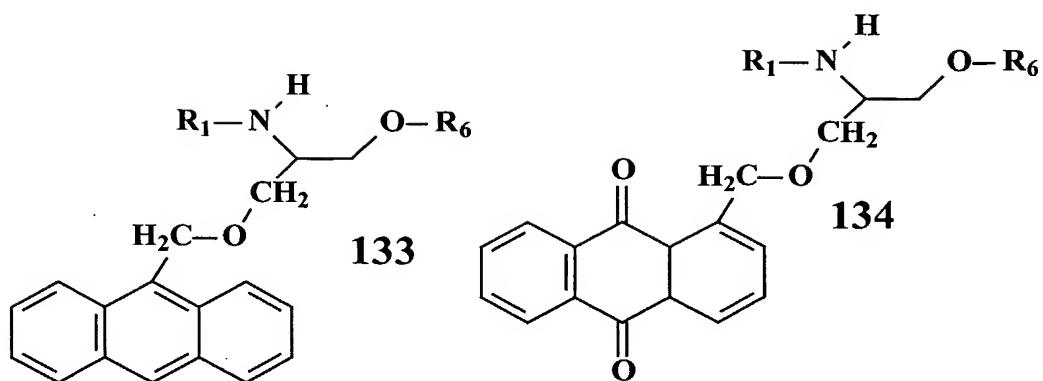


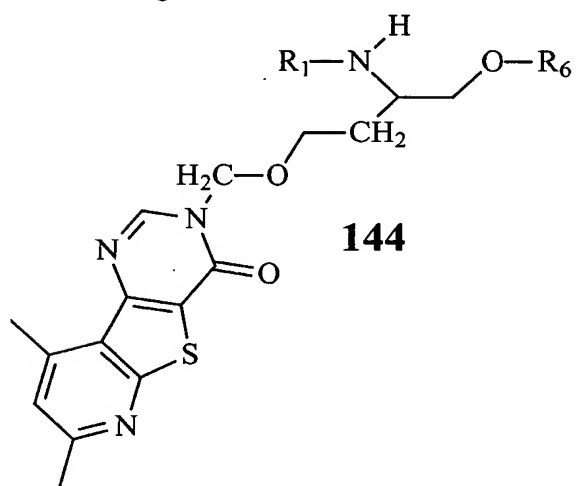
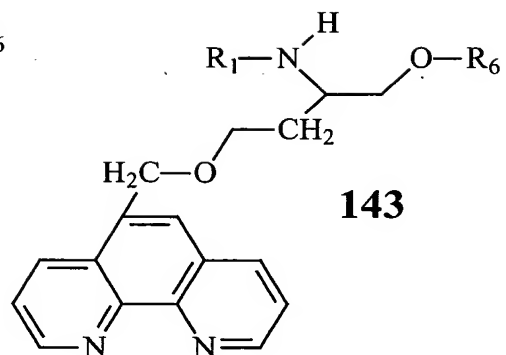
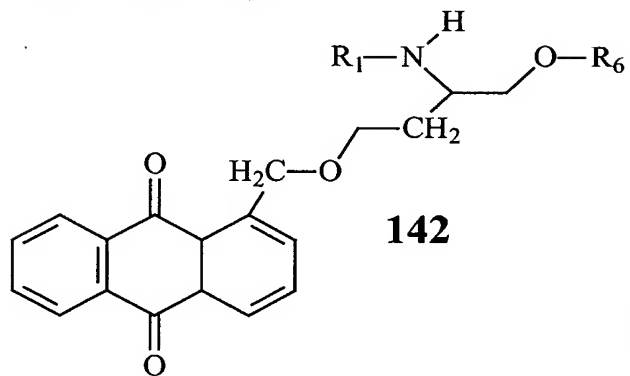
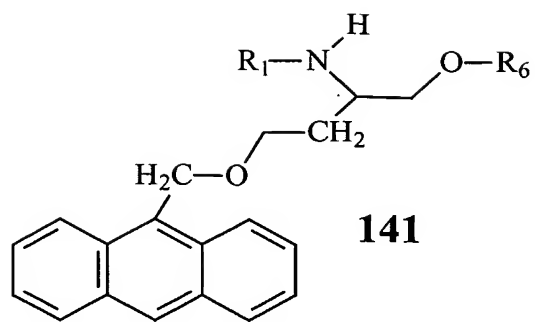
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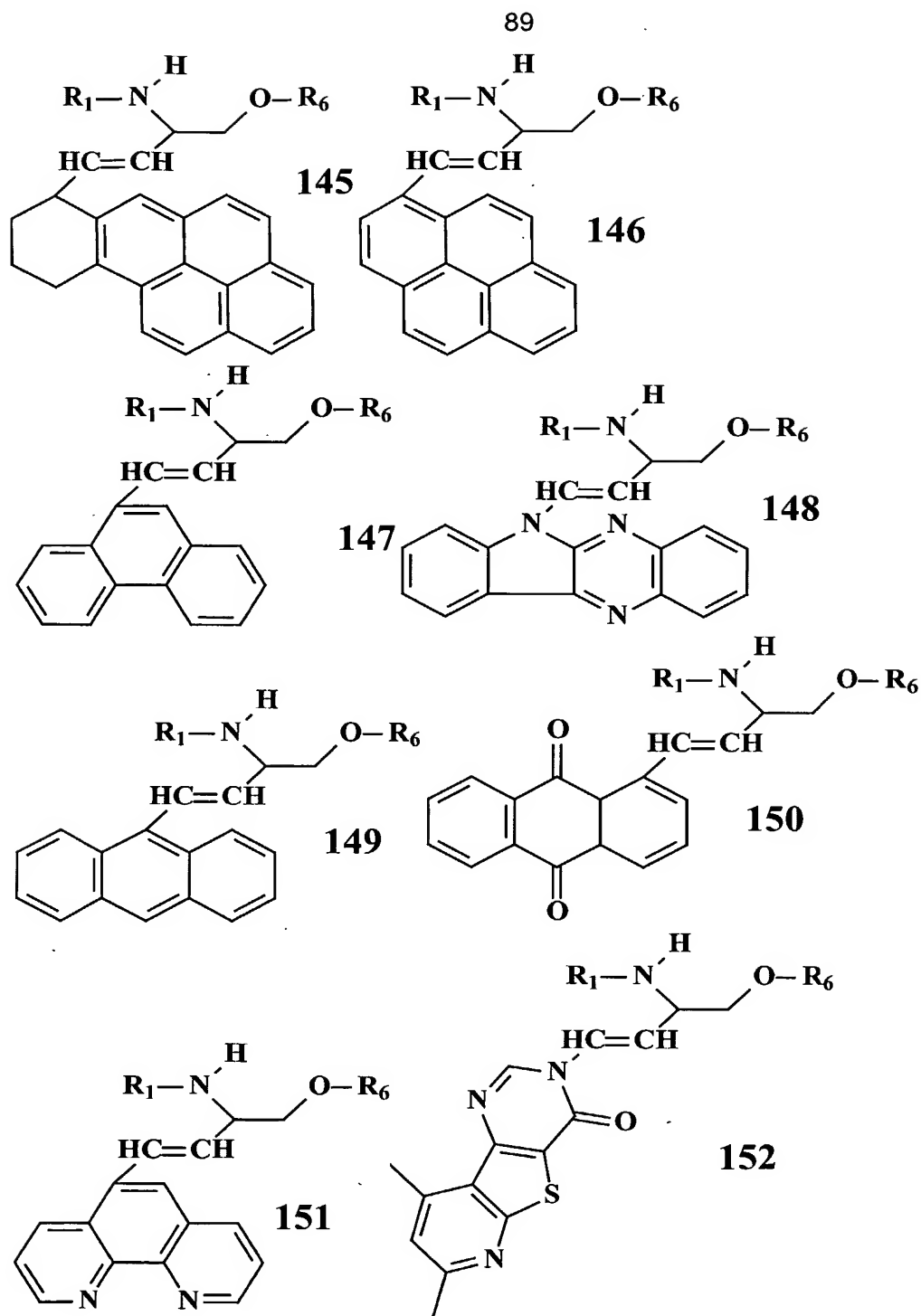


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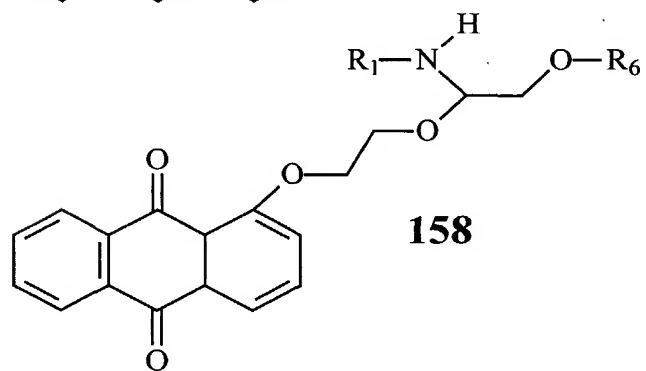
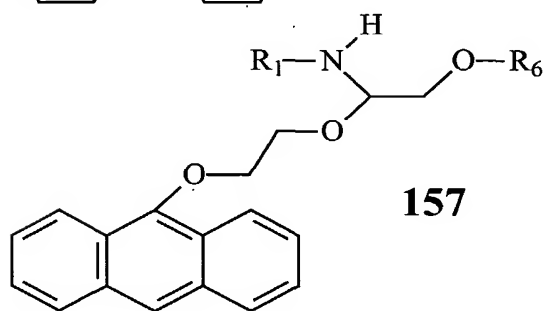
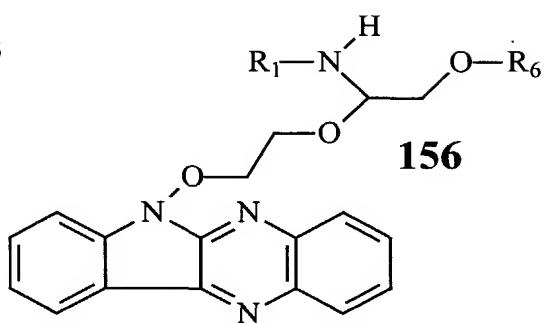
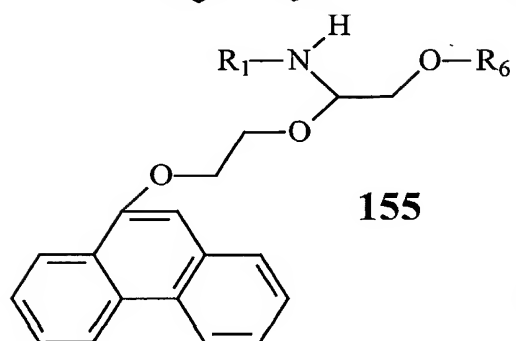
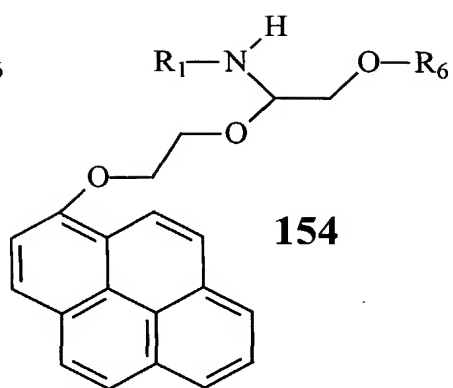
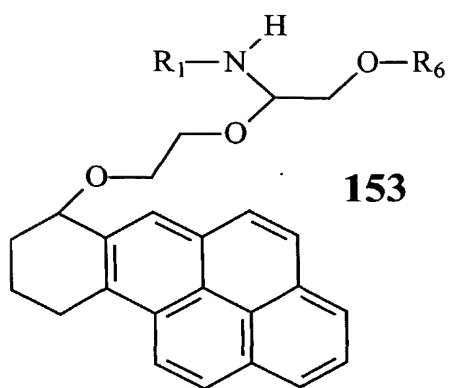


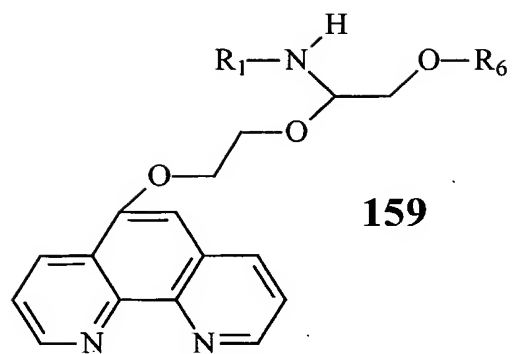
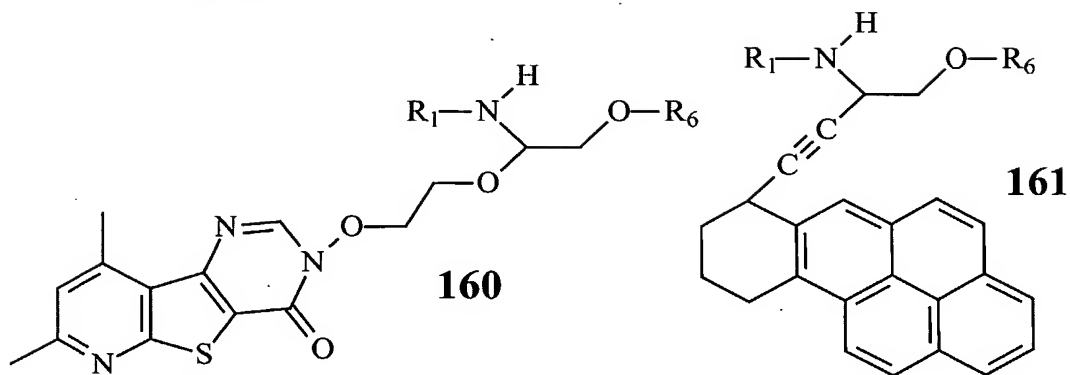
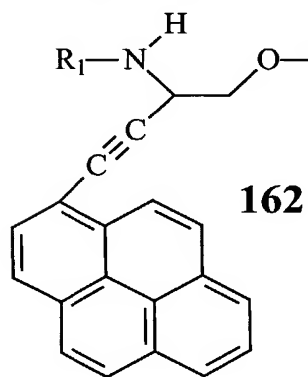
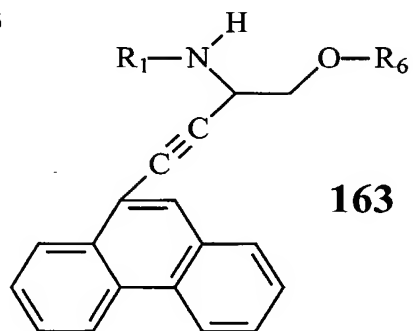
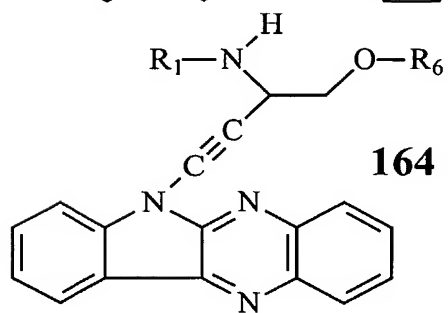
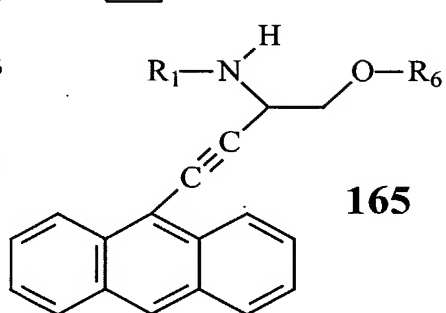




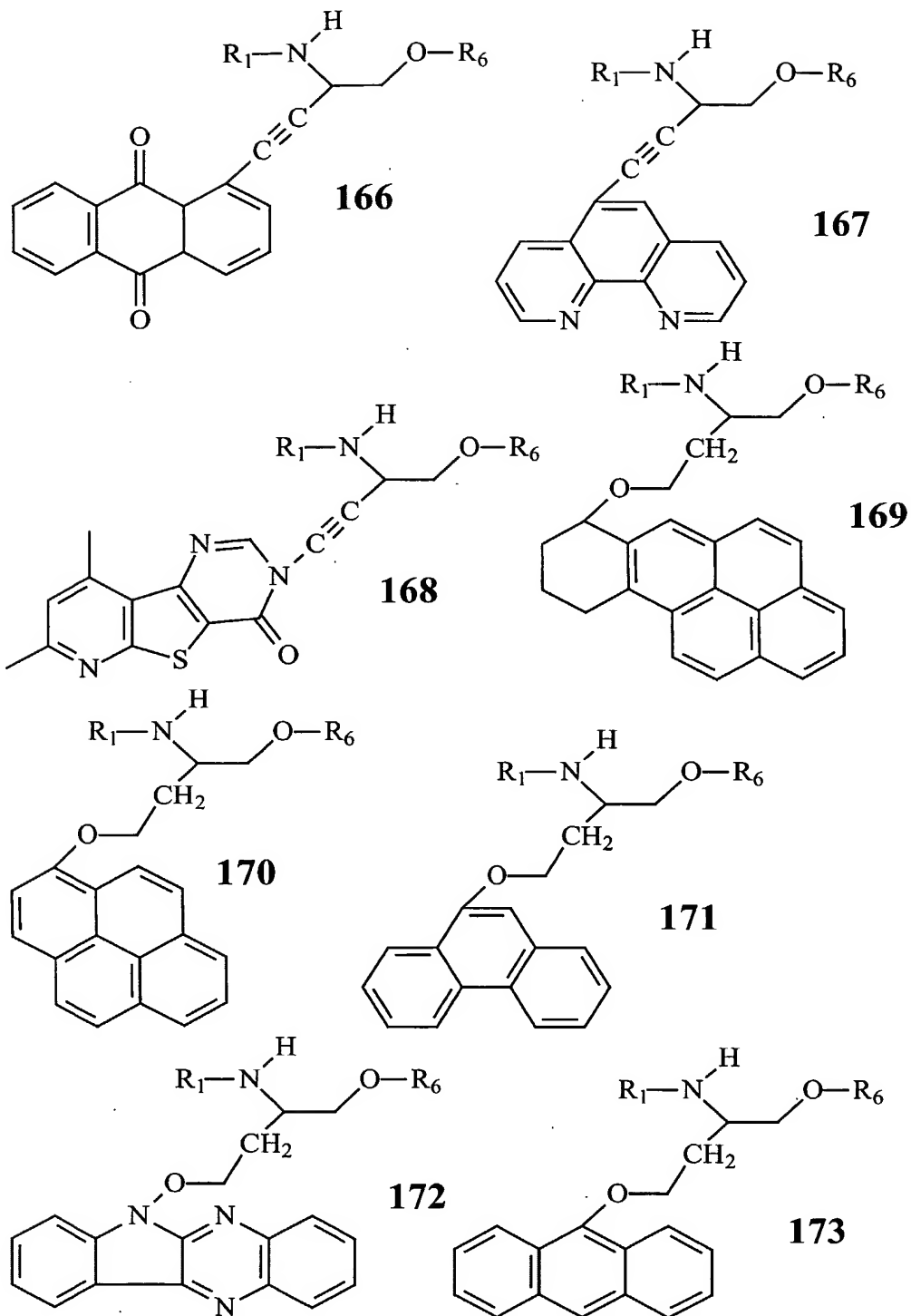


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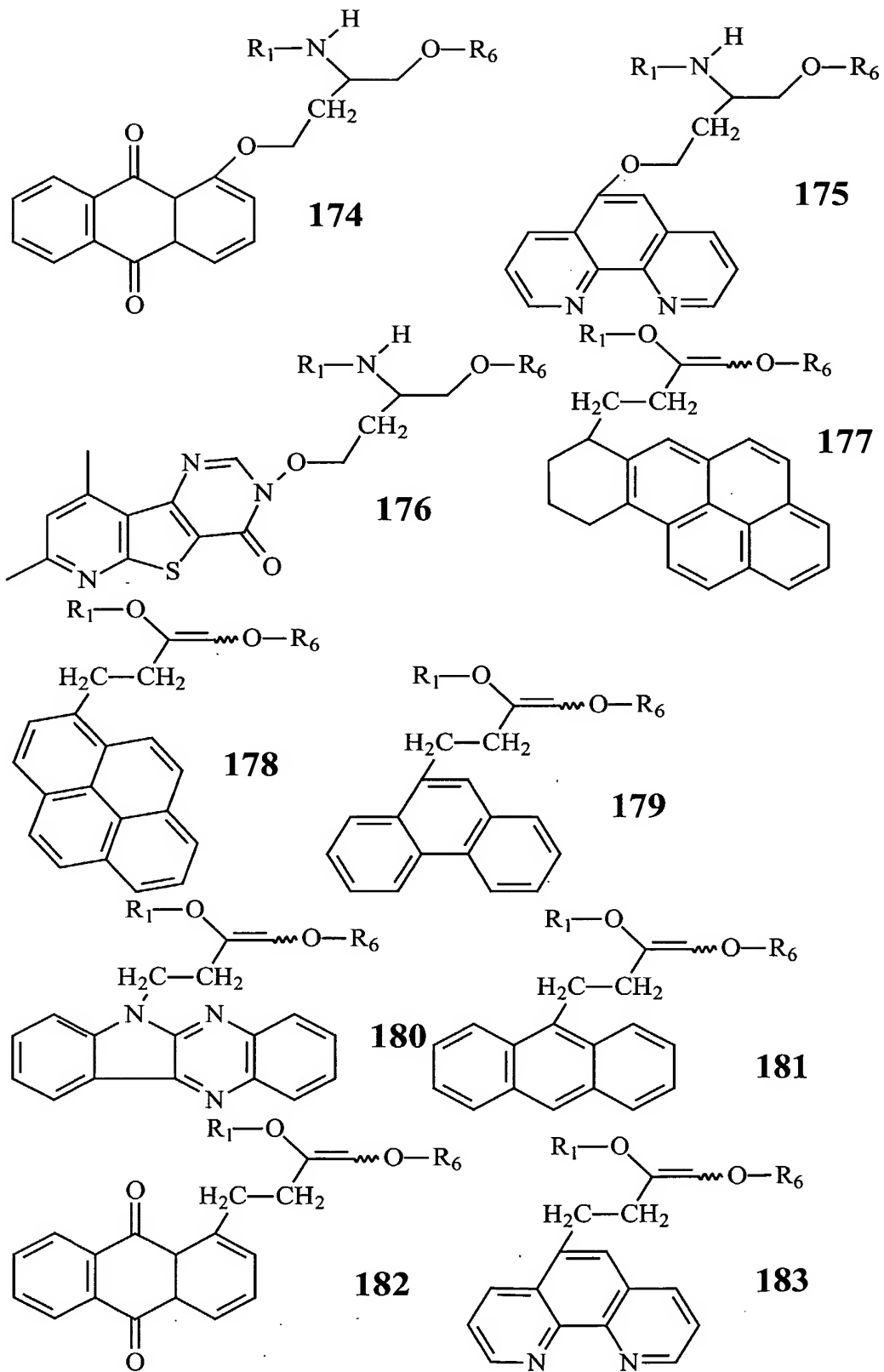


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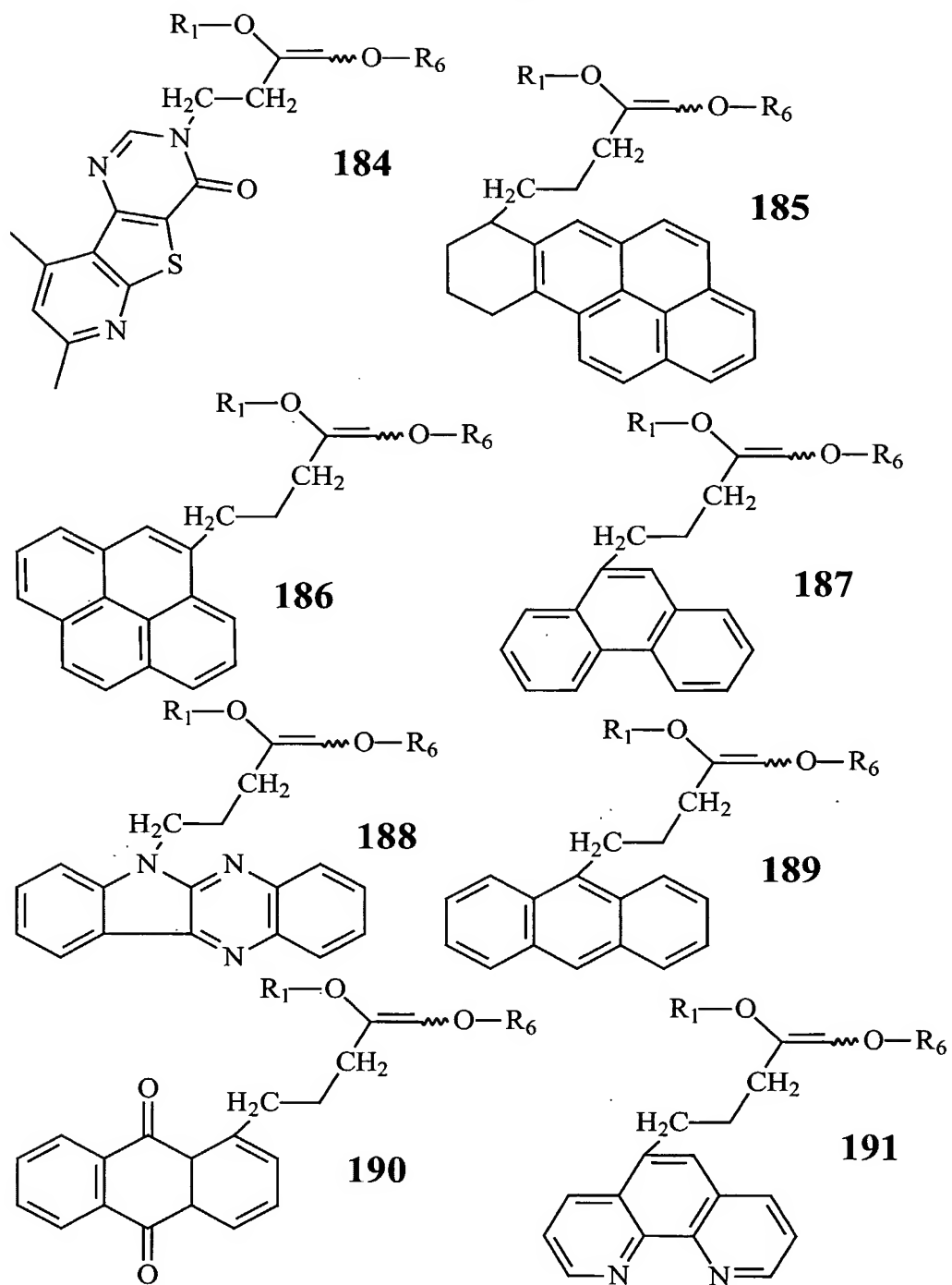


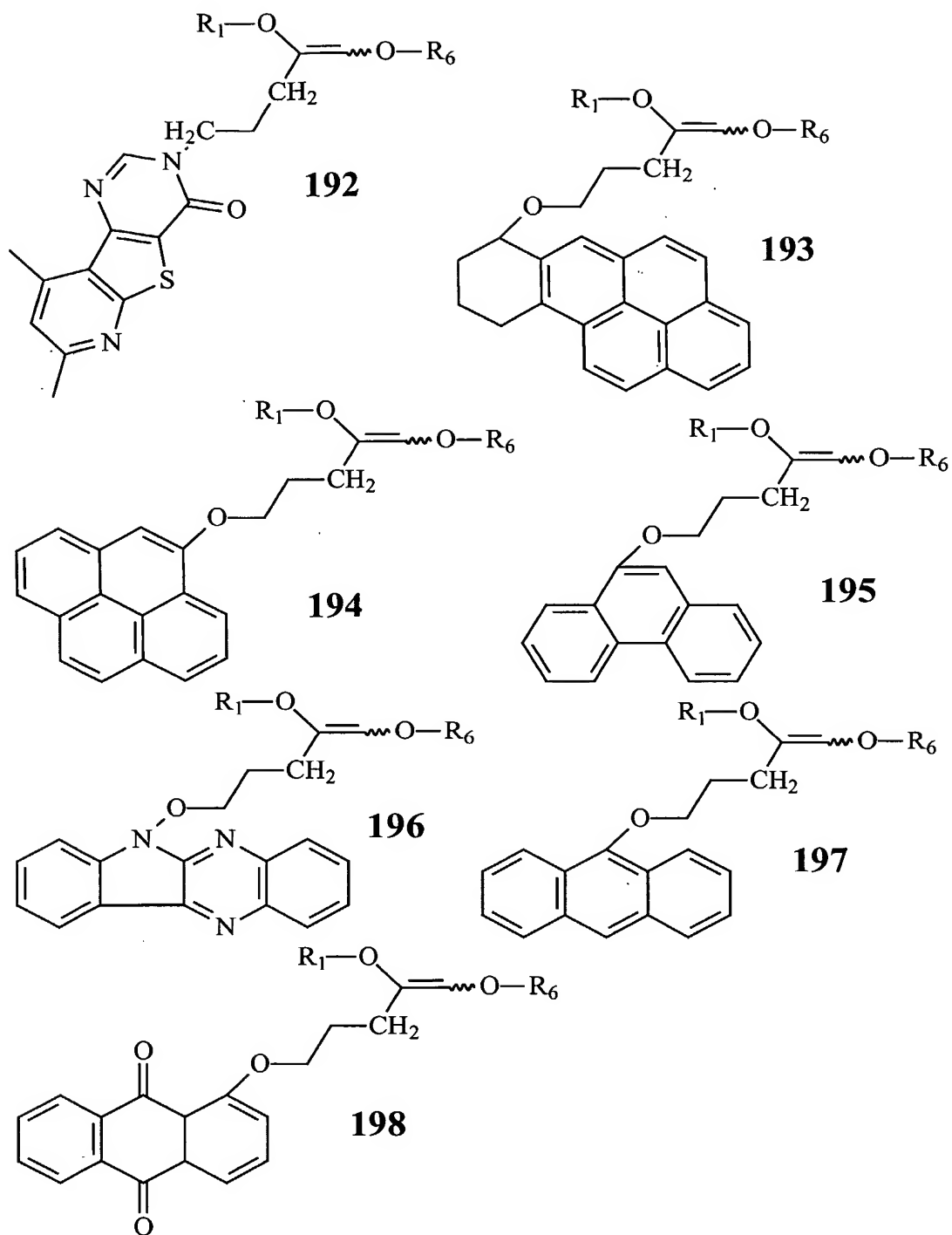


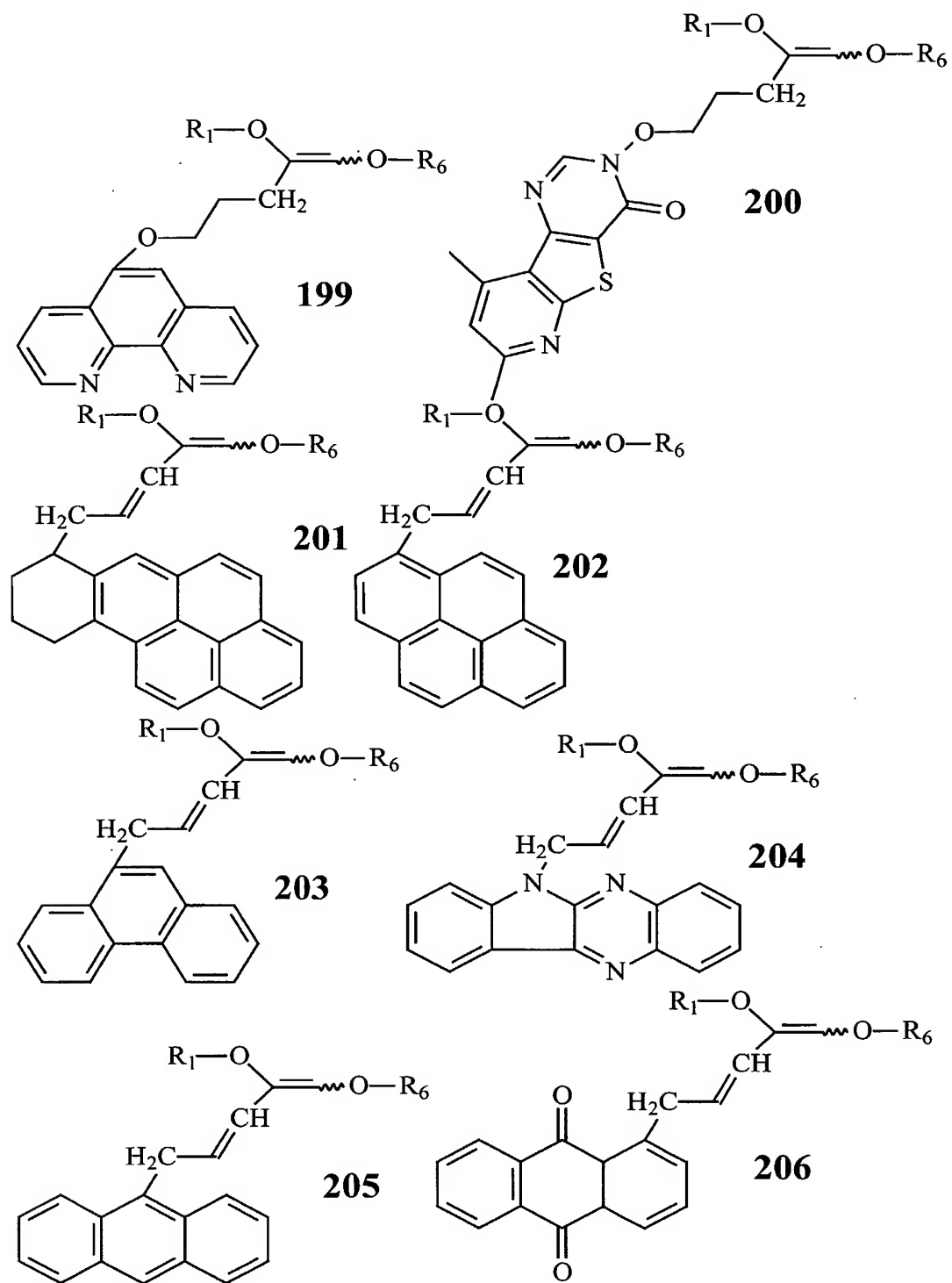
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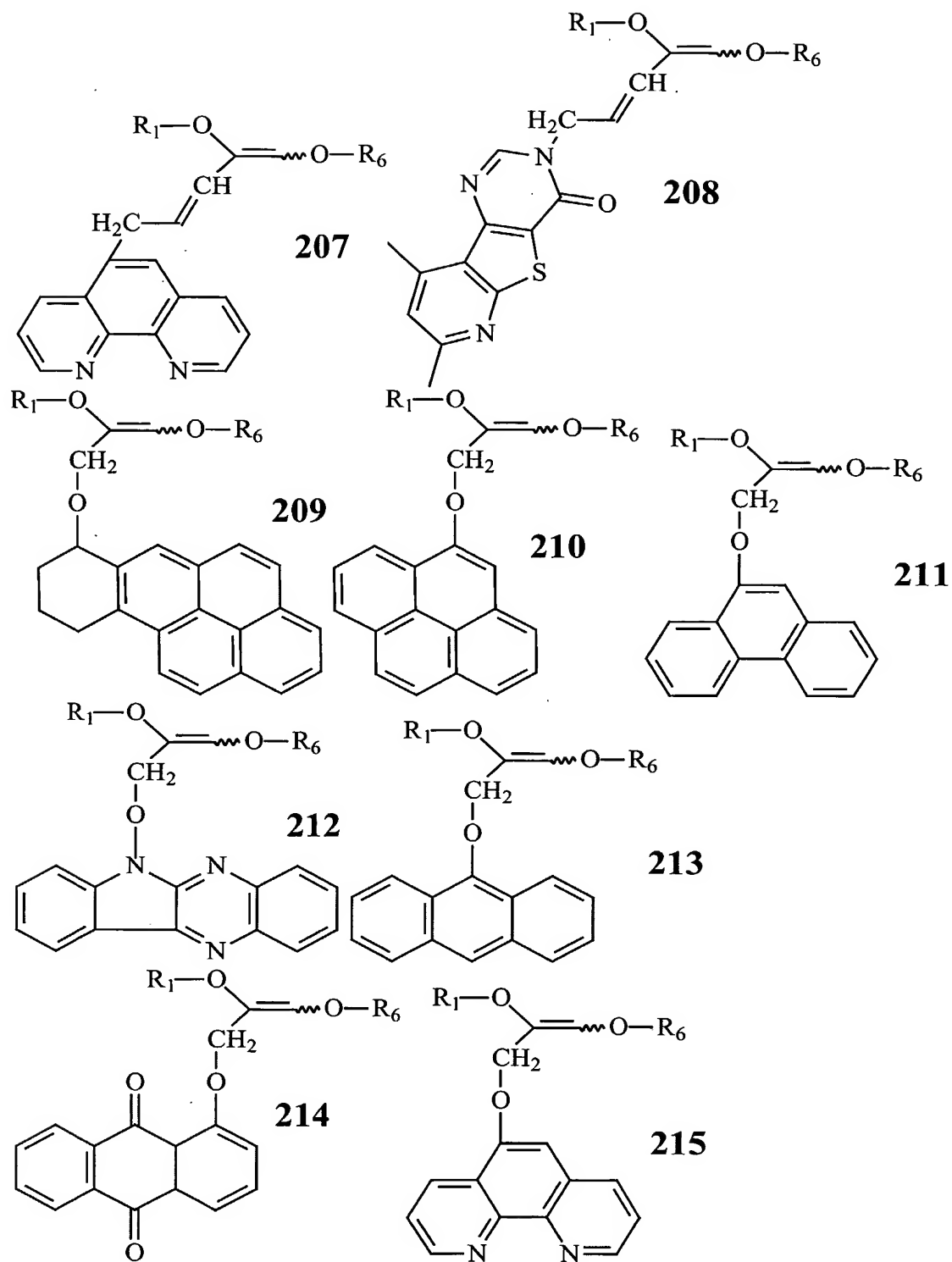
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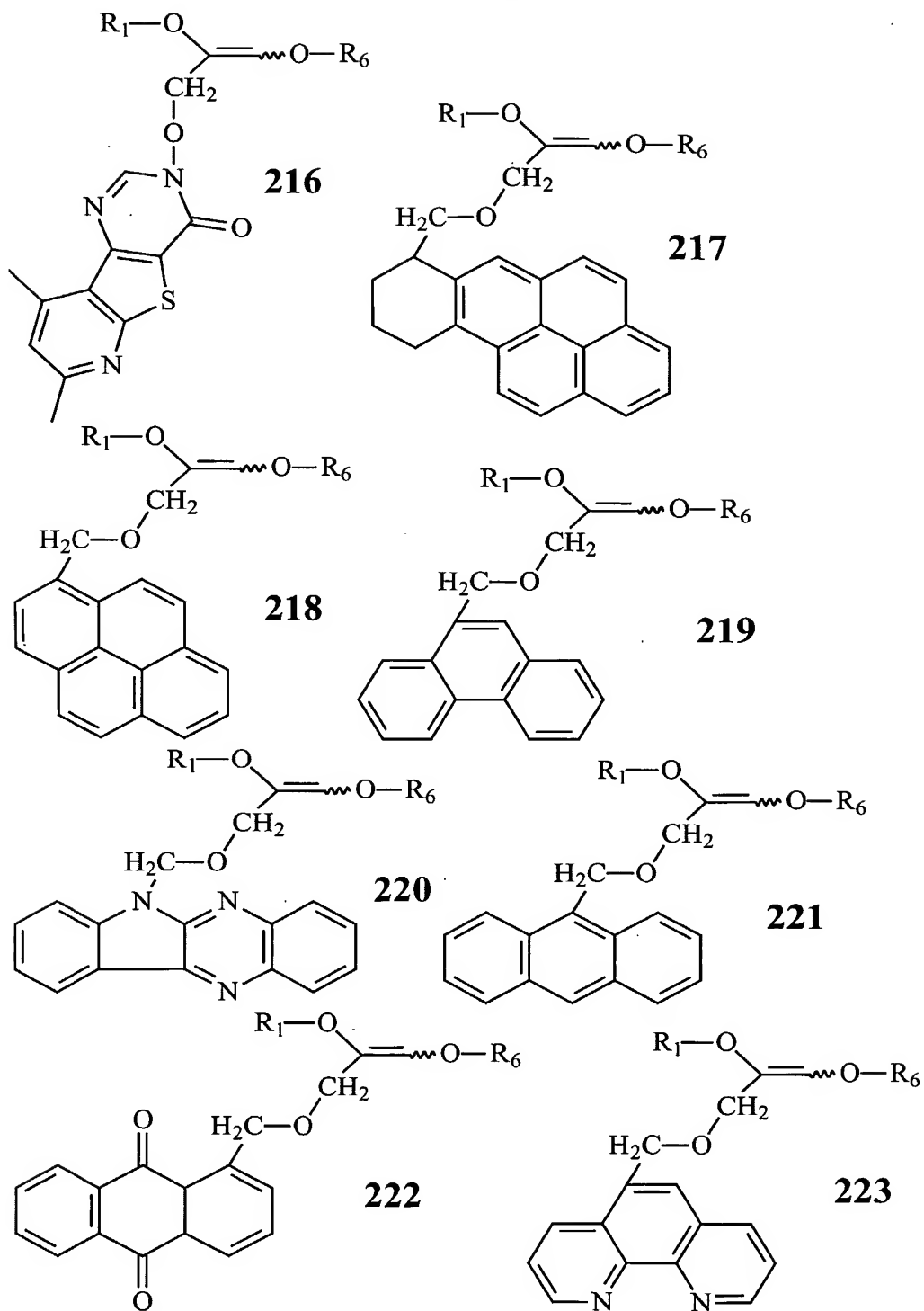


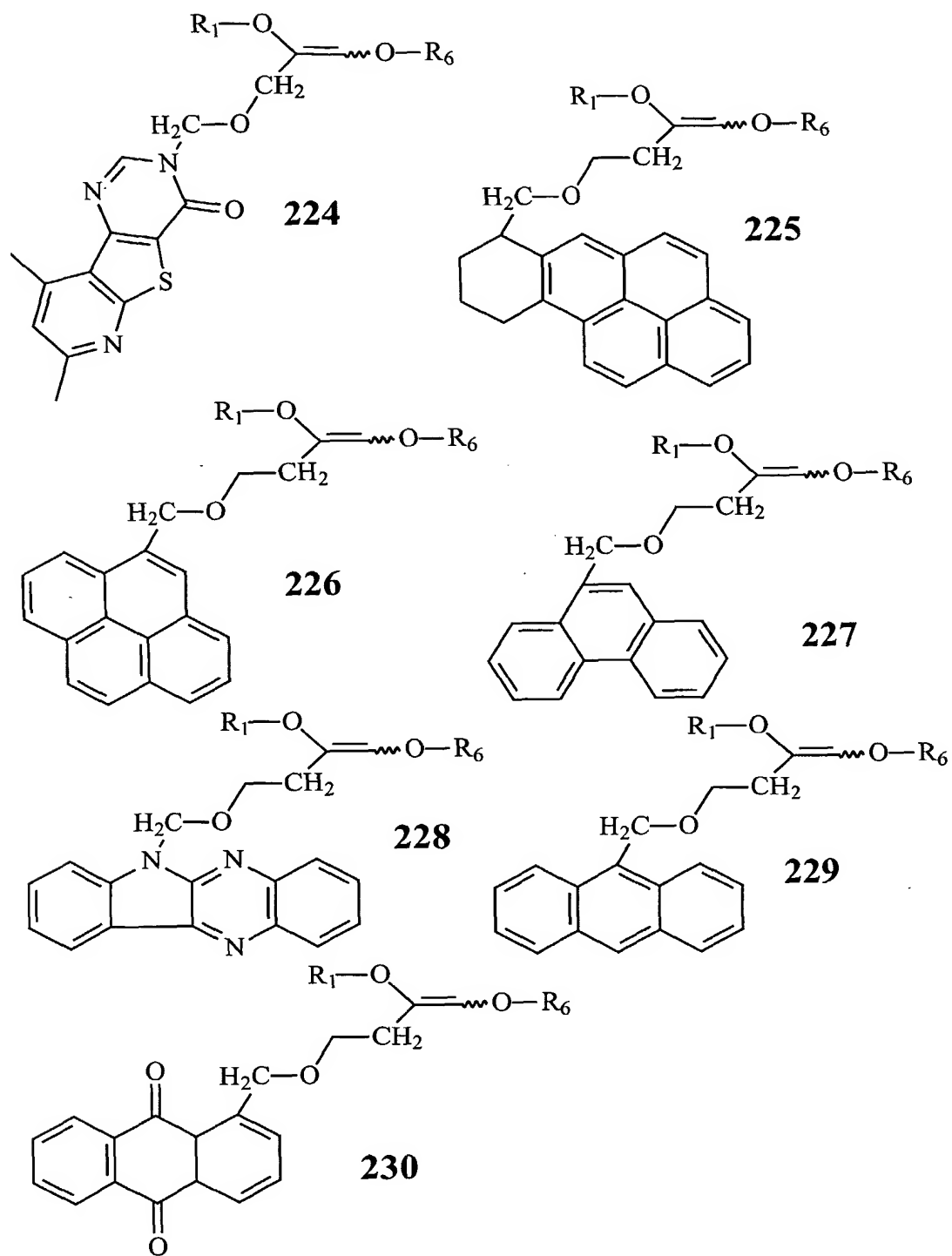




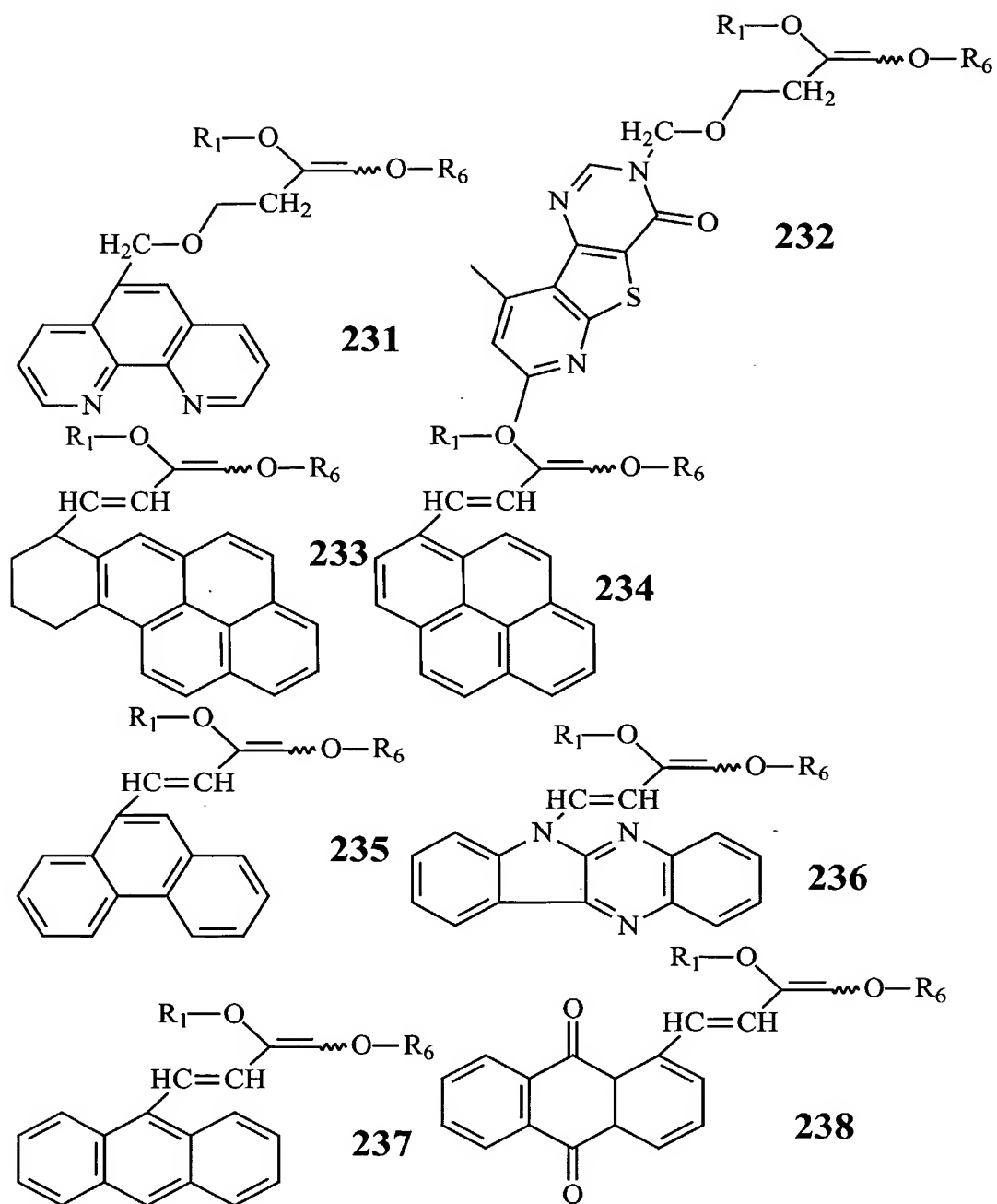
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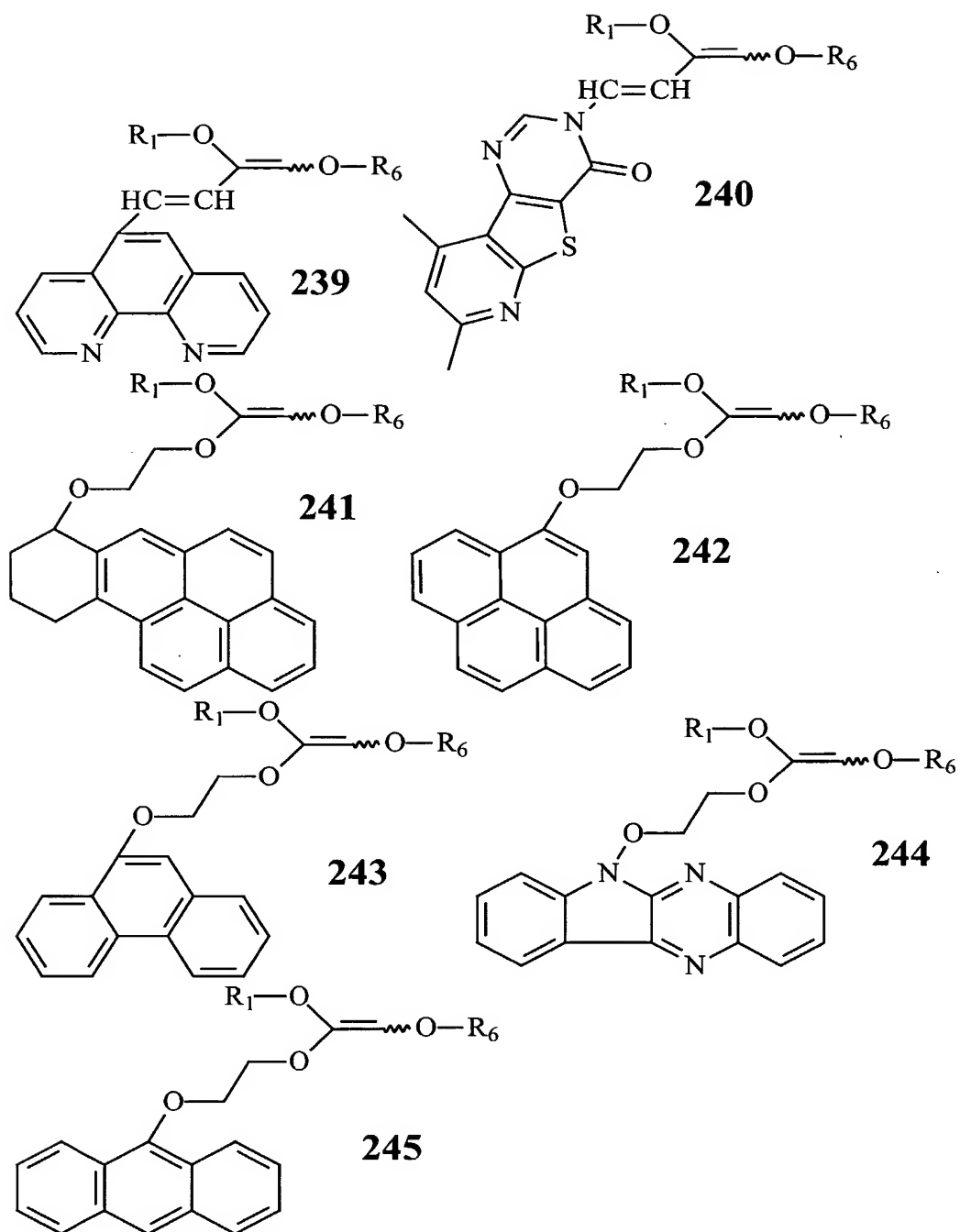




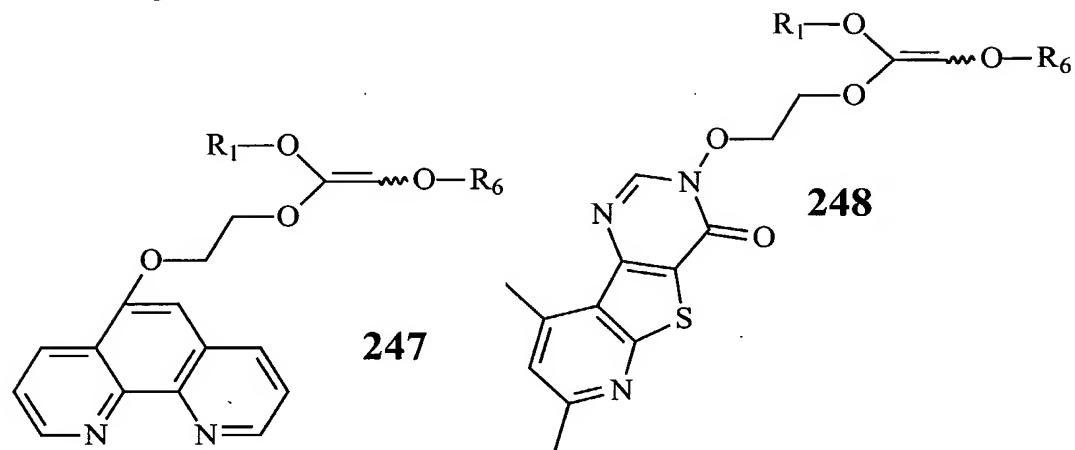
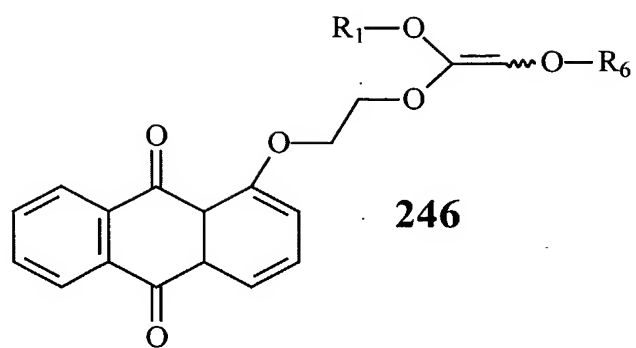




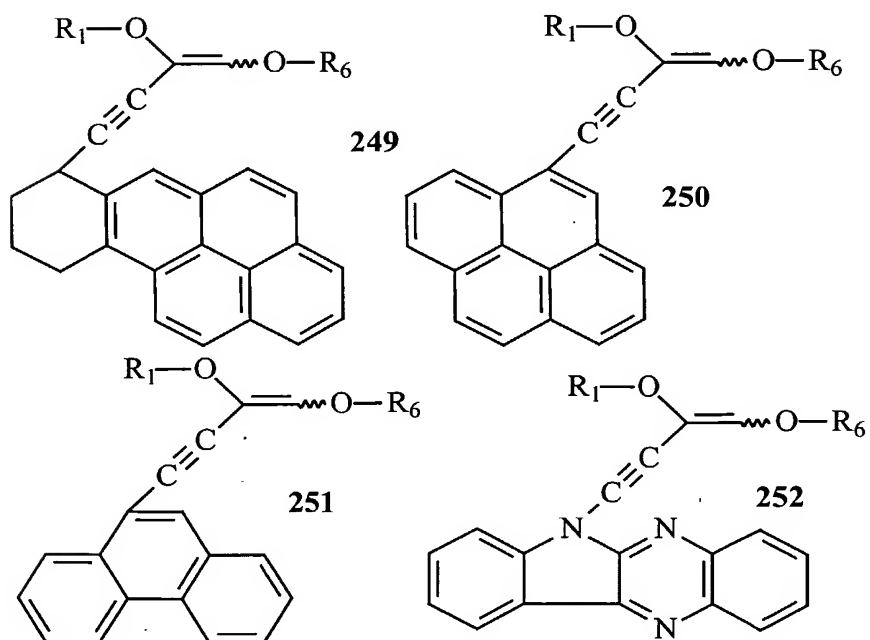
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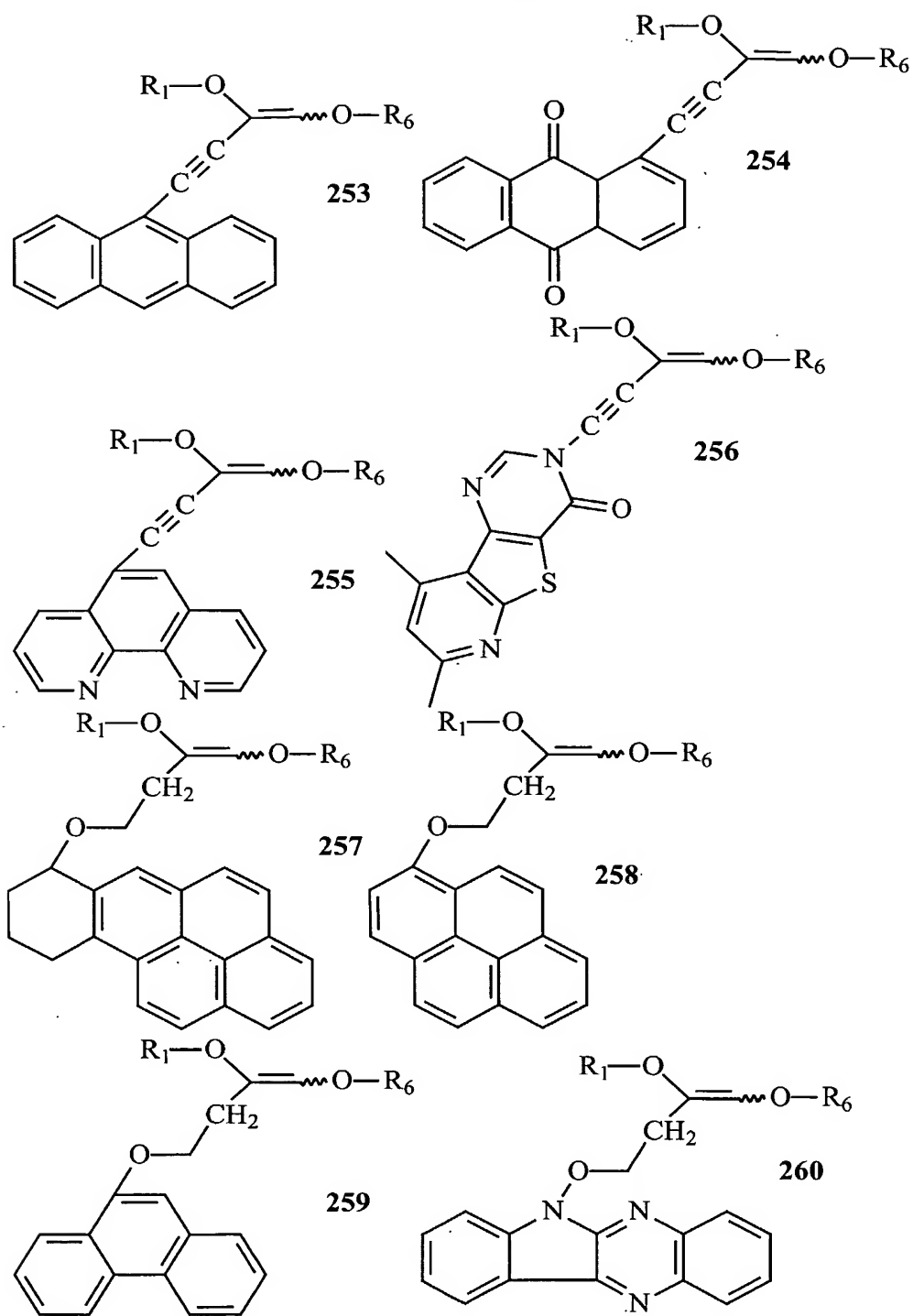
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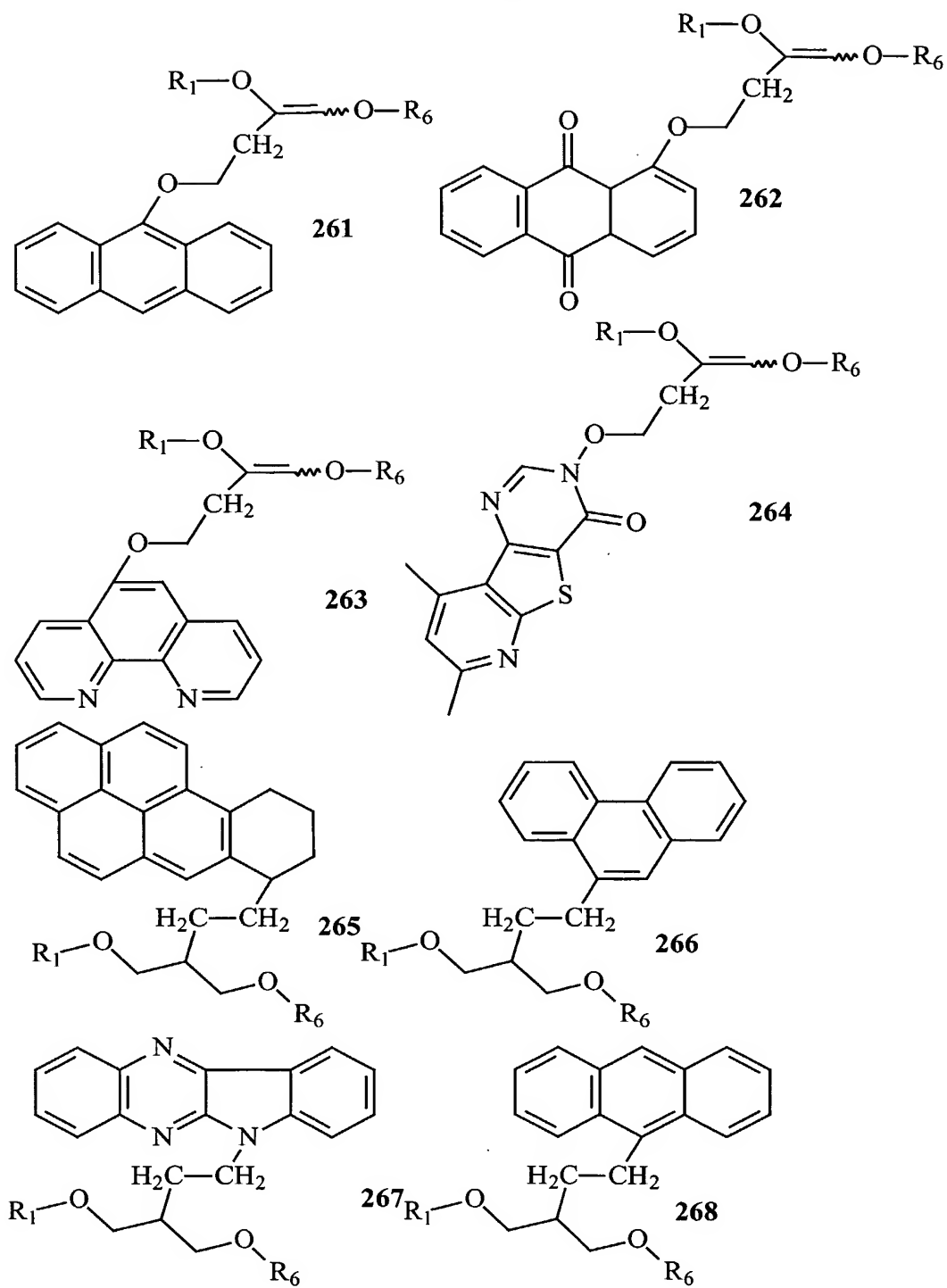
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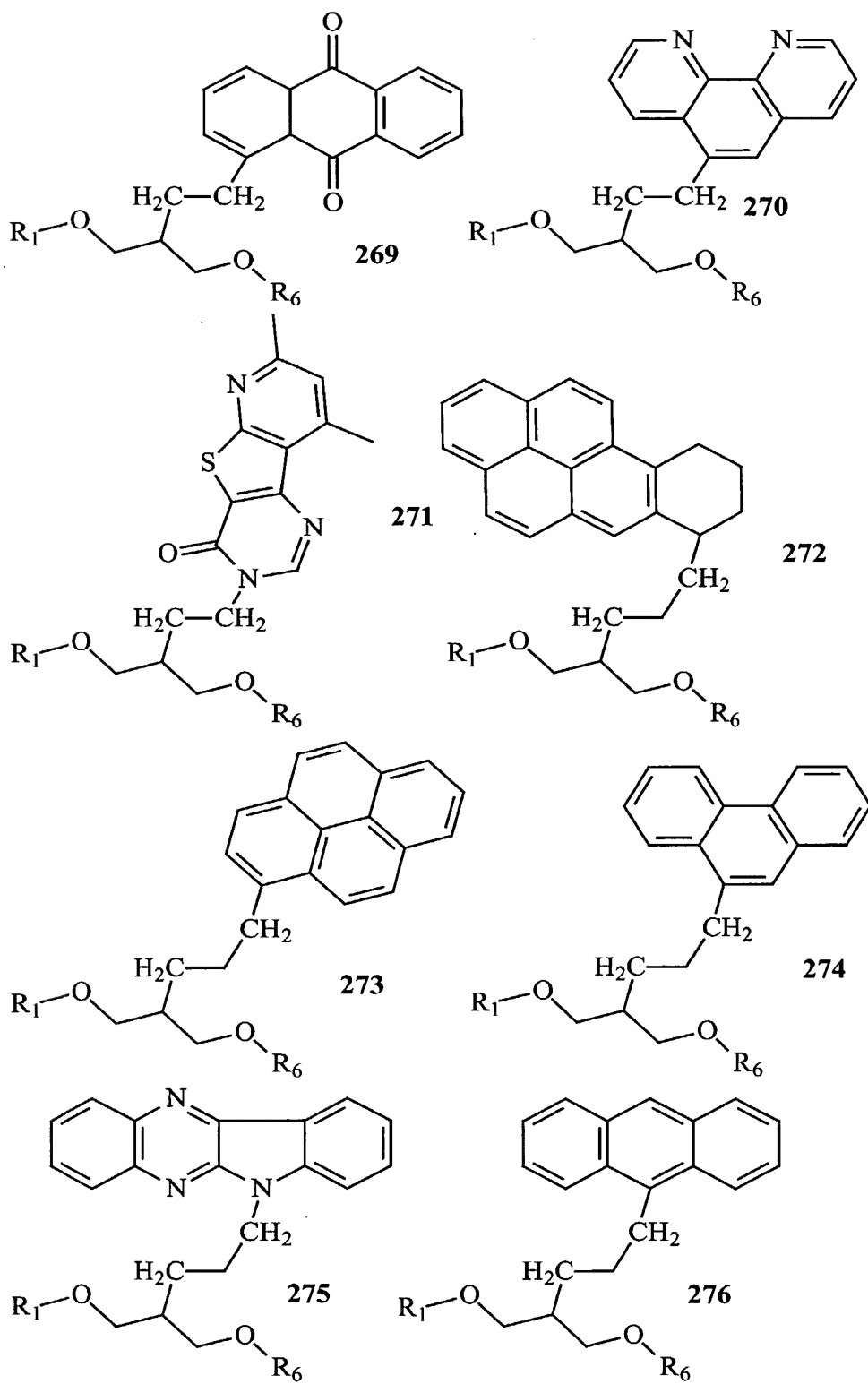
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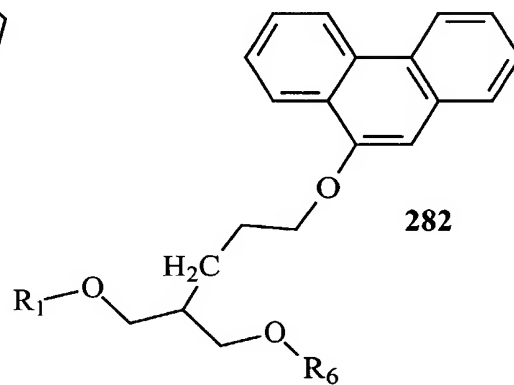
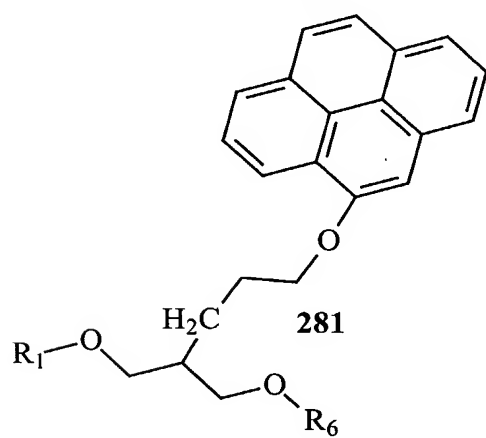
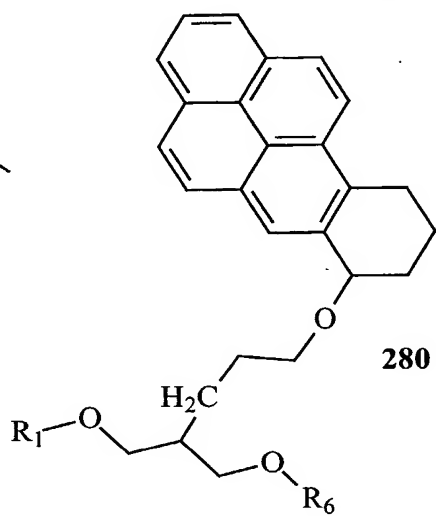
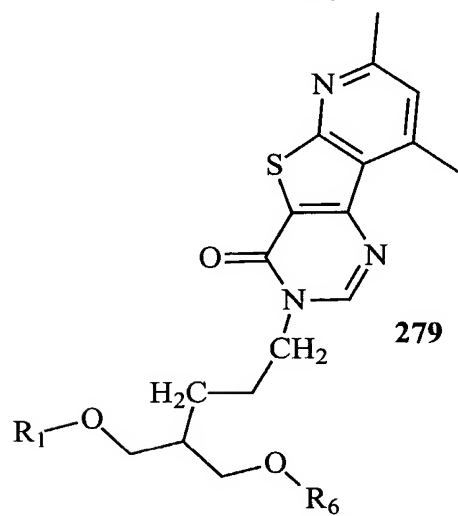
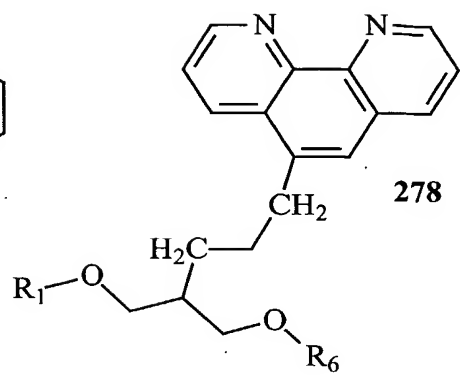
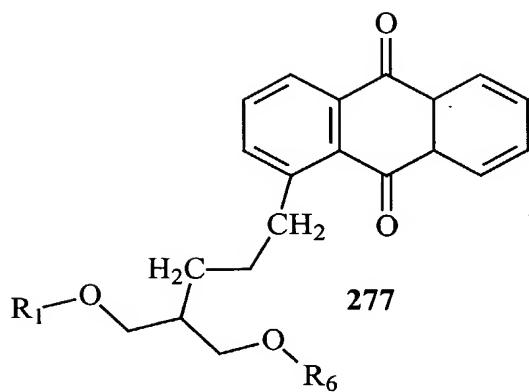


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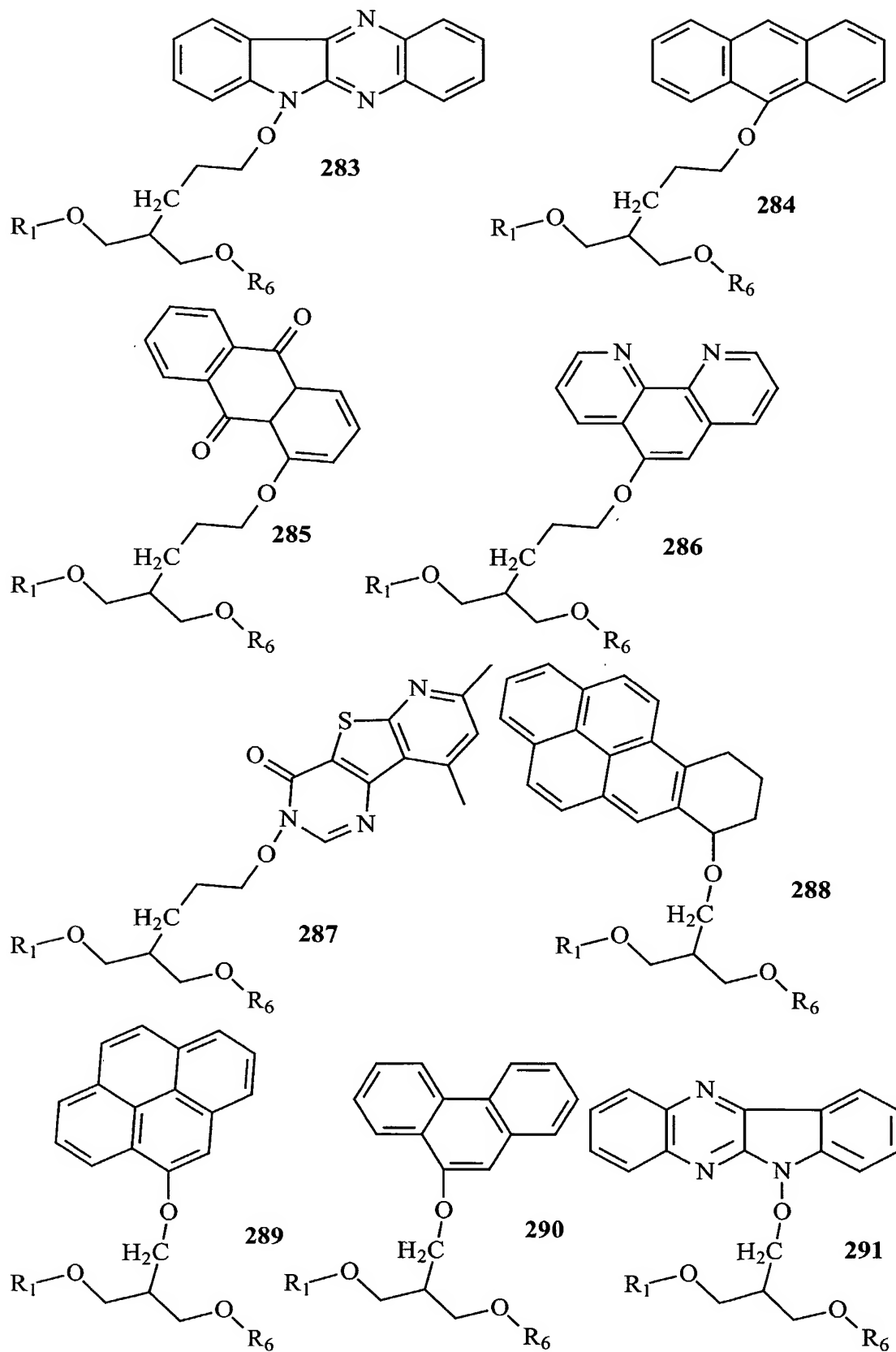


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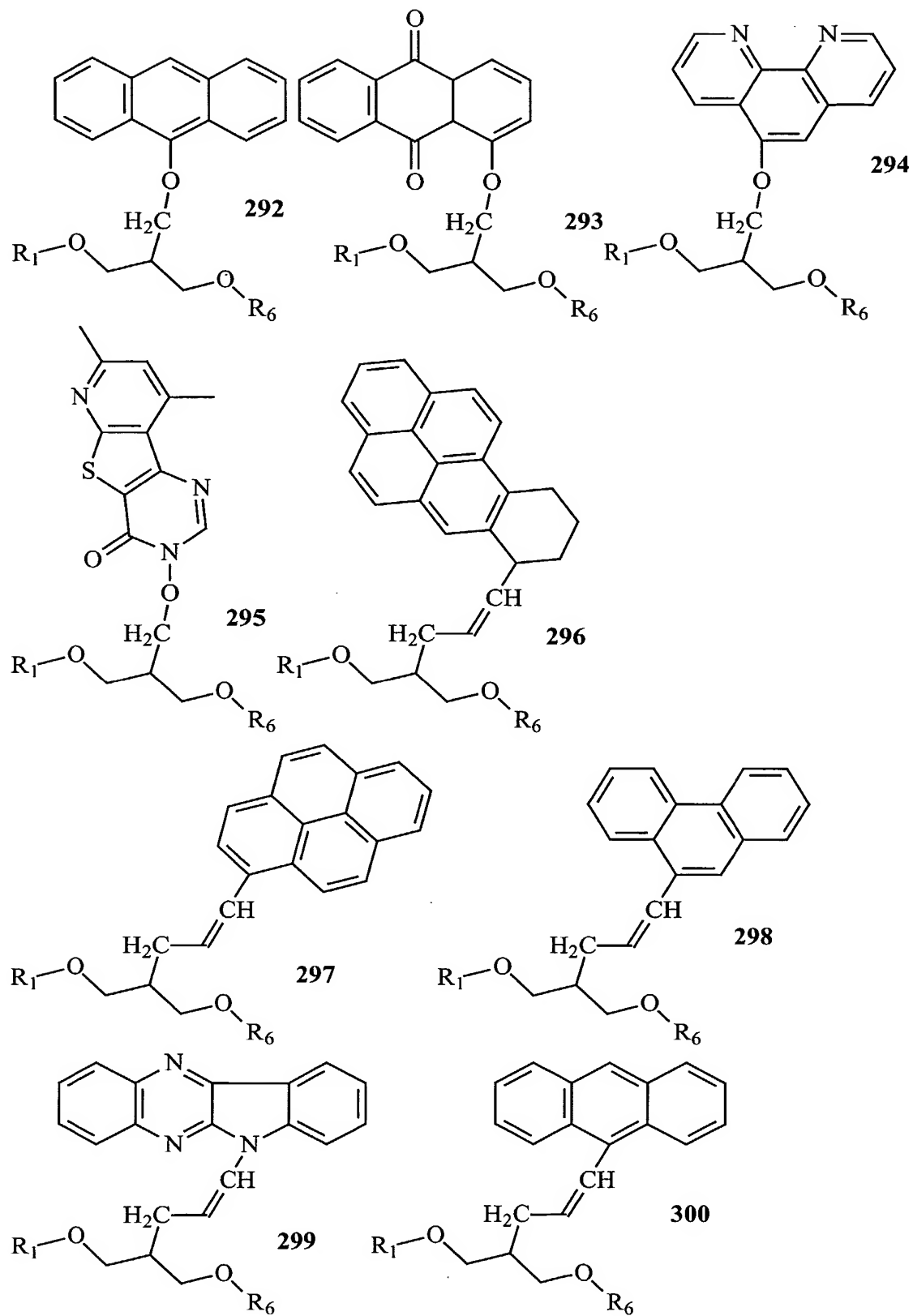


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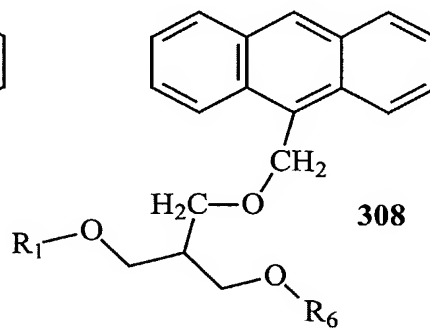
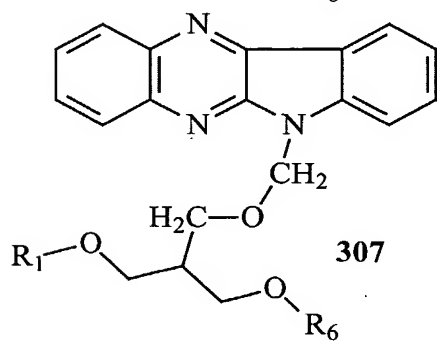
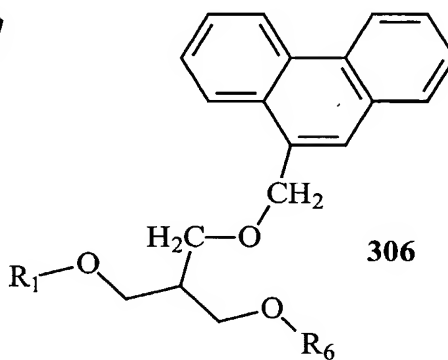
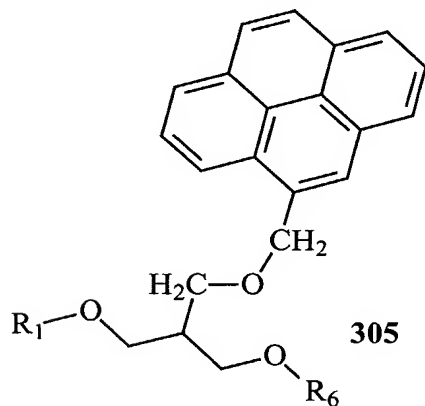
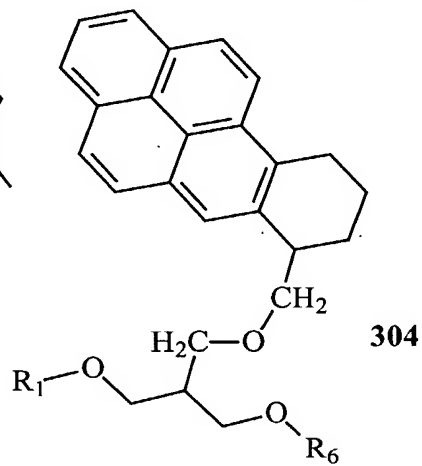
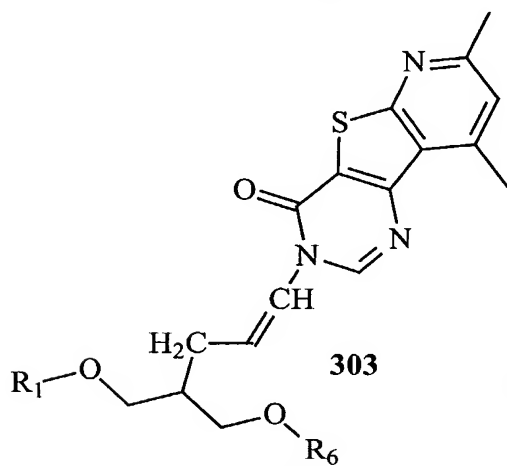
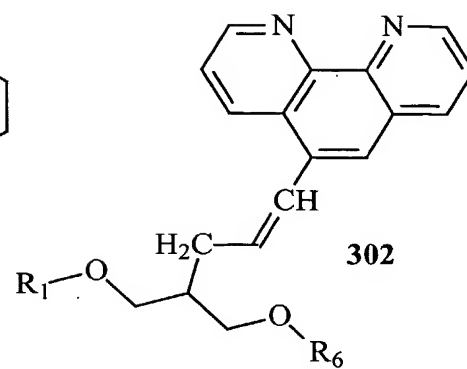
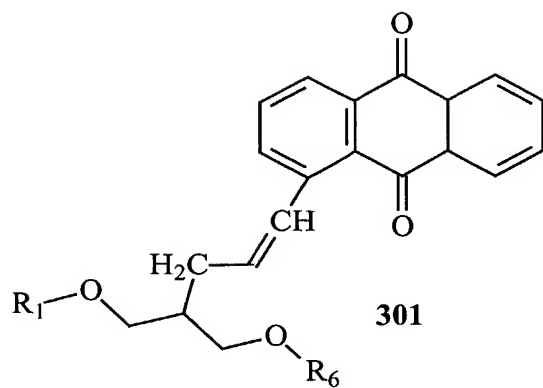




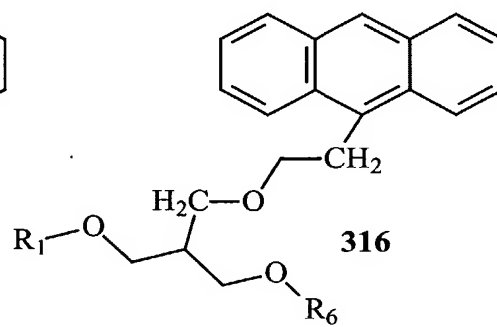
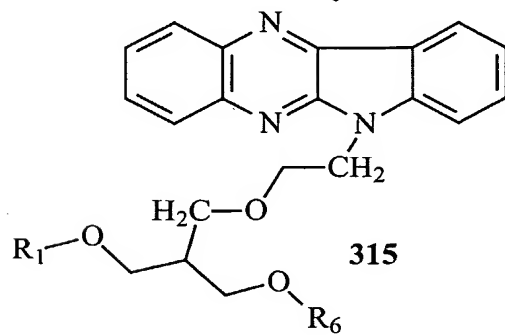
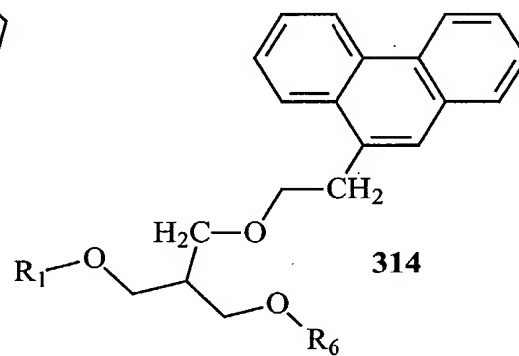
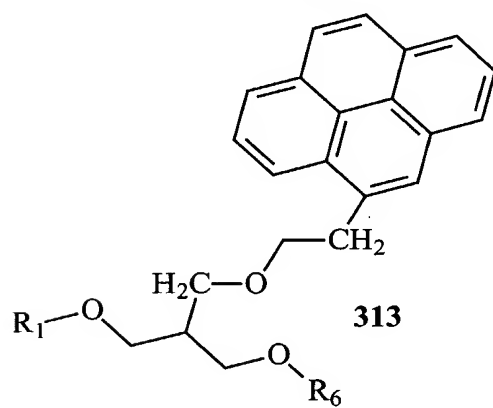
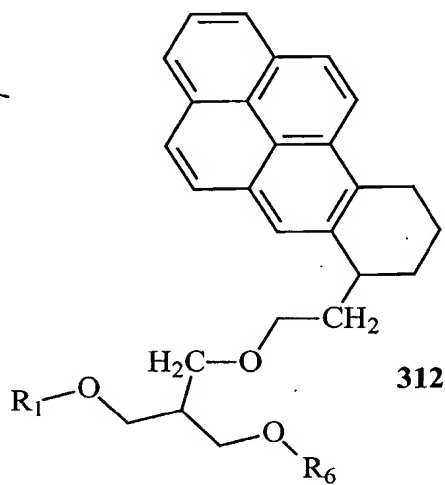
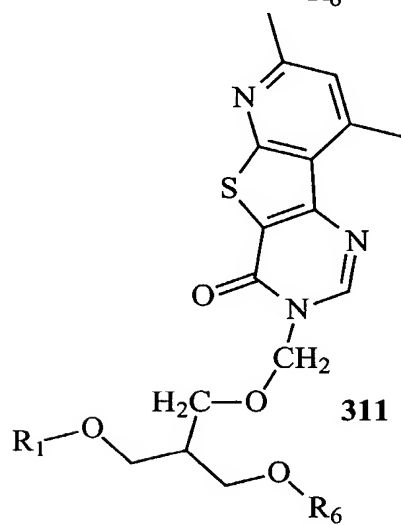
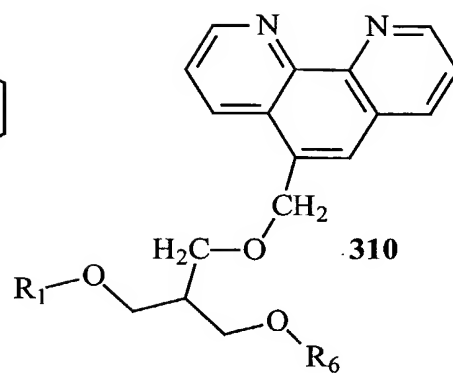
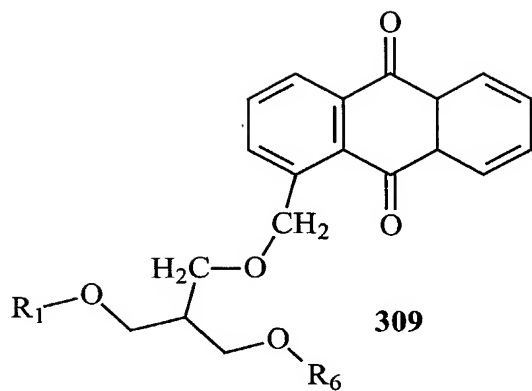
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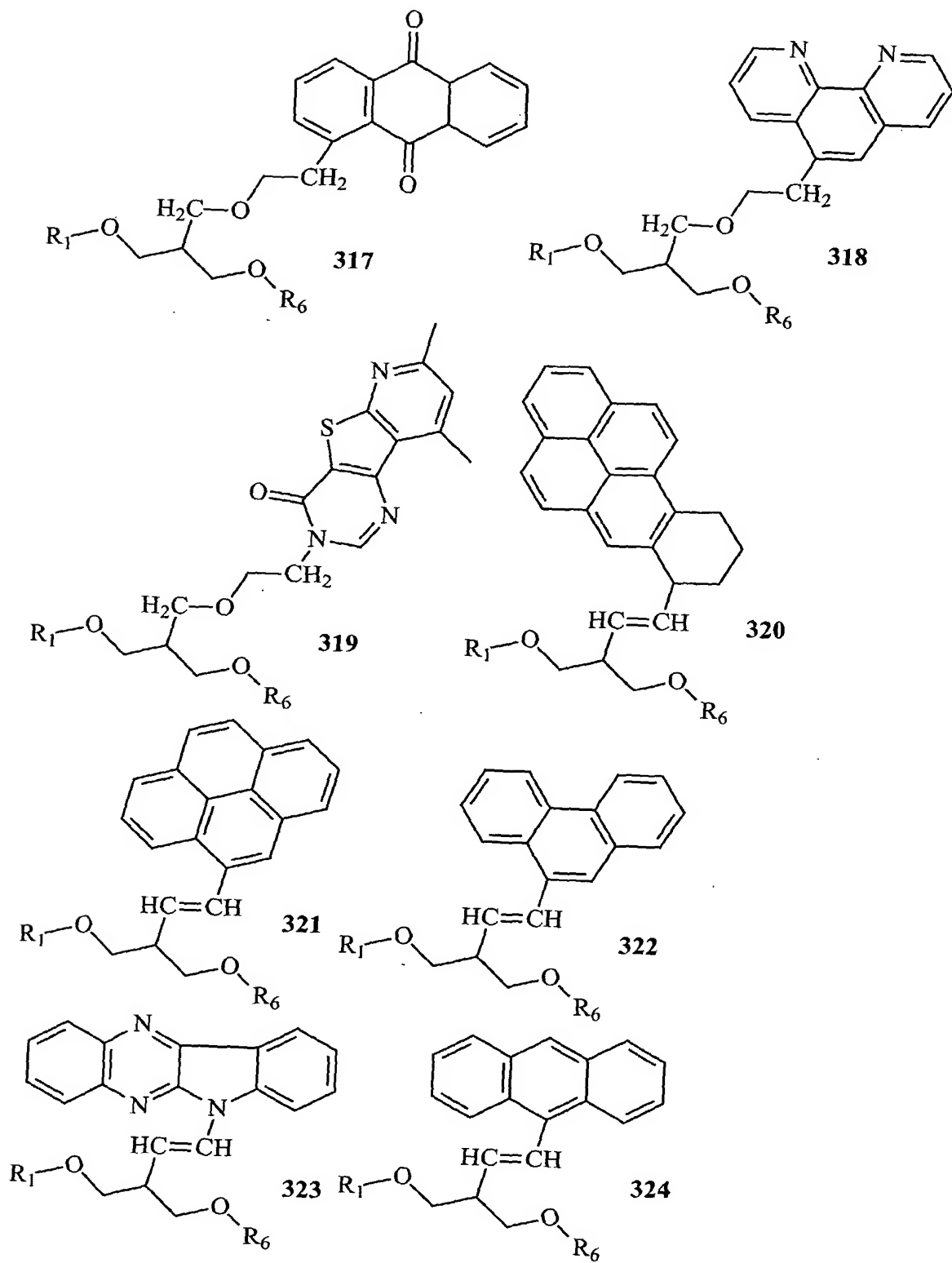
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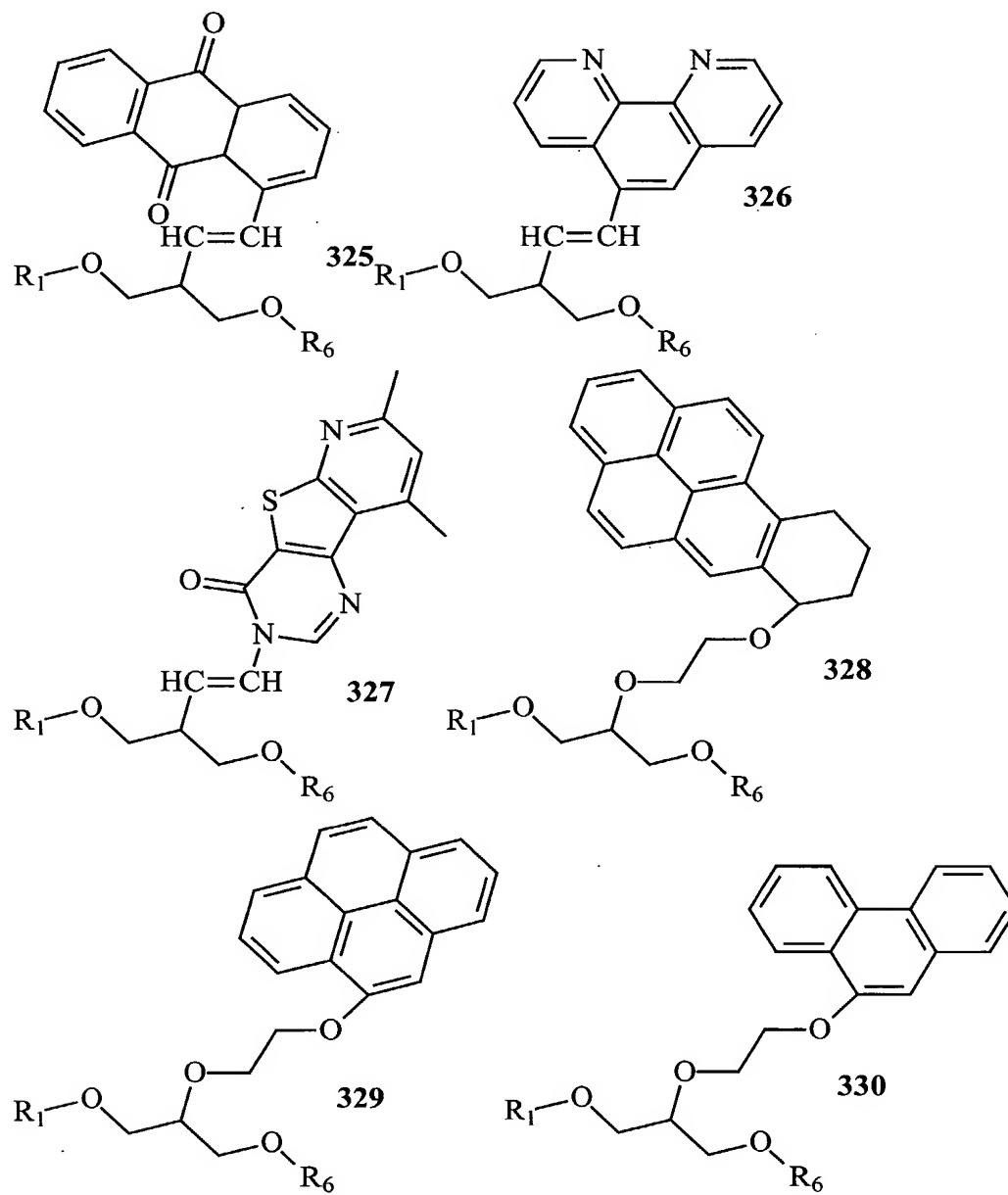
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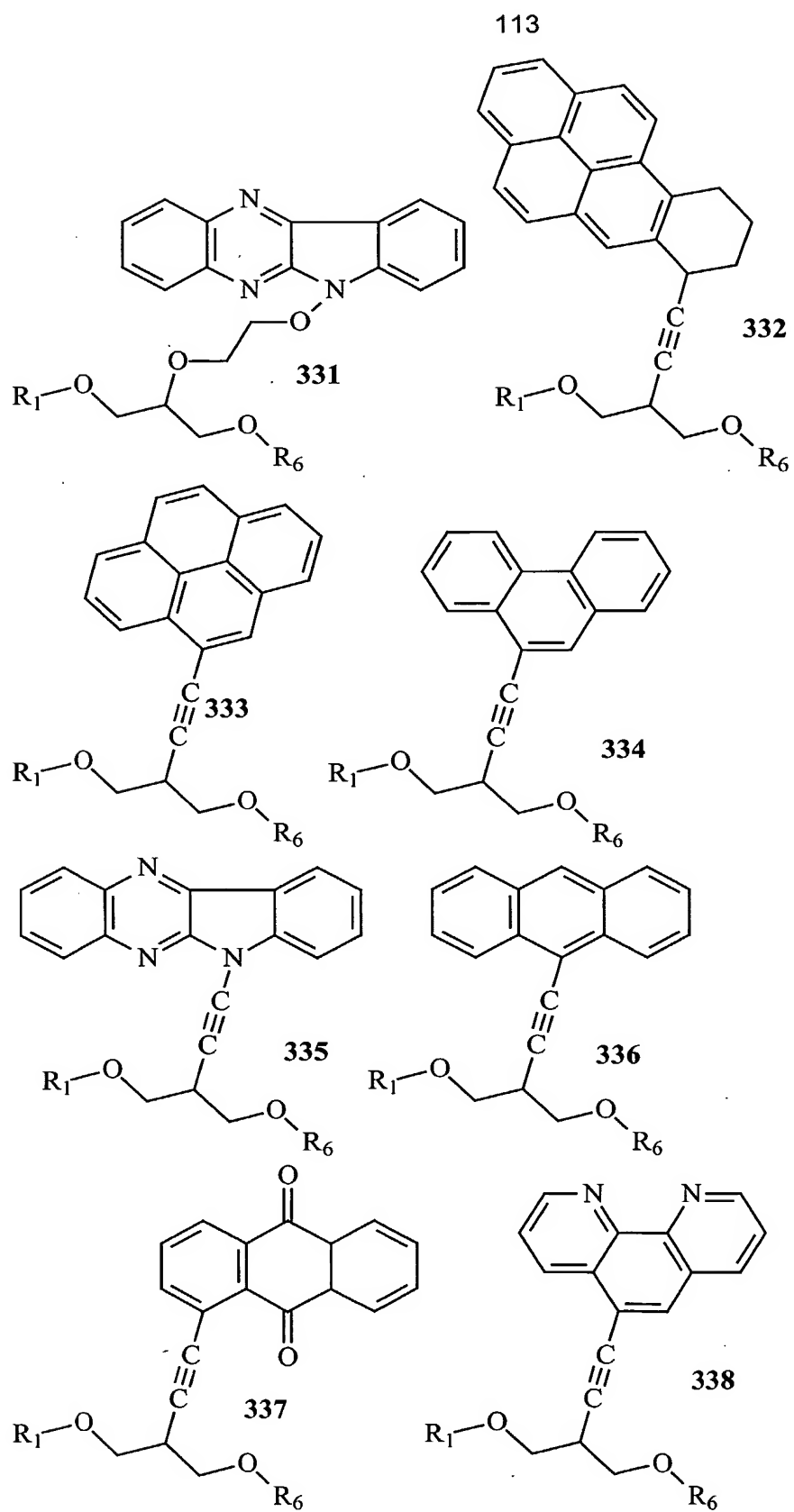


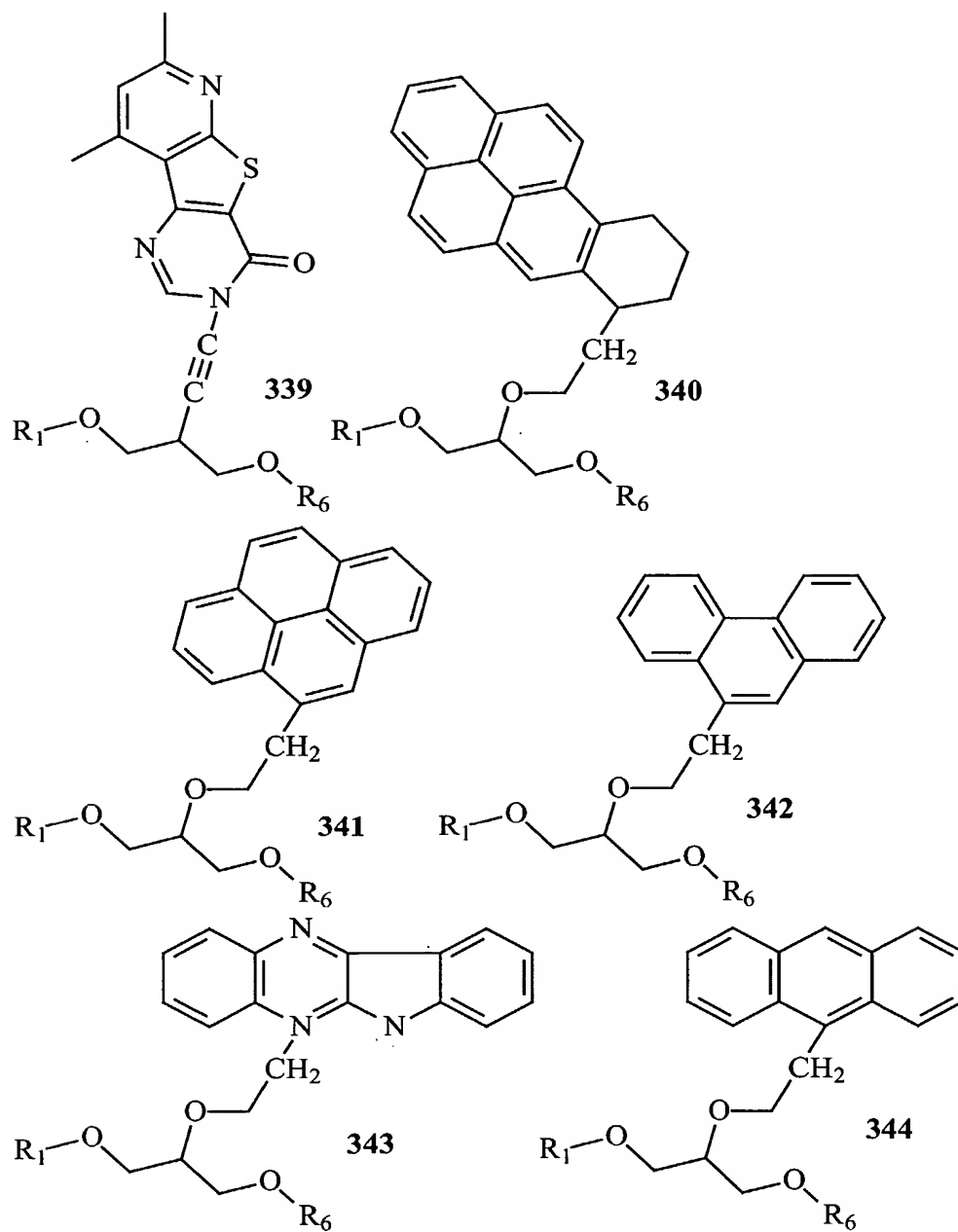
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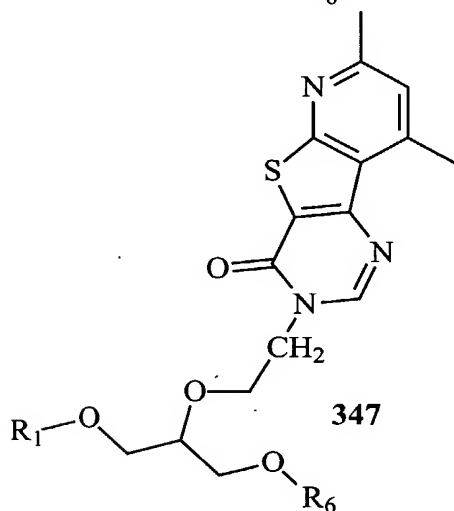
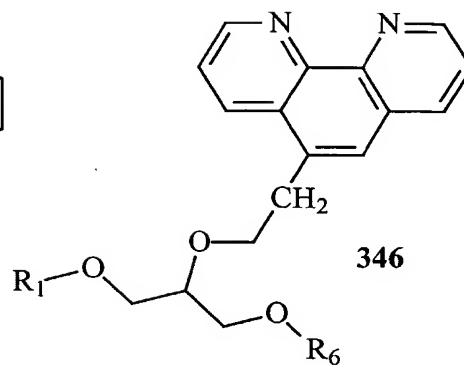
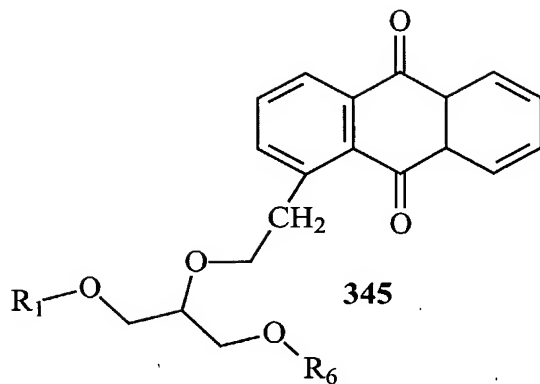
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5 wherein DMT and (CH<sub>2</sub>CH<sub>2</sub>CN) functions as protecting groups.

In one preferred embodiment of the present invention the intercalator pseudonucleotide is selected from the group consisting of phosphoramidites of 1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol. Even more preferably, the intercalator pseudonucleotide is selected from the group consisting of the phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol and the phosphoramidite of (R)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol.

#### Preparation of intercalator pseudonucleotides

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The intercalator pseudonucleotides according to the present invention may be synthesised by any suitable method.



However preferably the method may comprise the steps of

5 a1) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and optionally a linker part coupled to a reactive group; and

10 b1) providing a linker precursor molecule comprising at least two reactive groups, said two reactive groups may optionally be individually protected; and

c1) reacting said intercalator with said linker precursor and thereby obtaining an intercalator-linker; and

15 d1) providing a backbone monomer precursor unit comprising at least two reactive groups, said two reactive groups may optionally be individually protected and/or masked) and optionally comprising a linker part; and

20 e1) reacting said intercalator-linker with said backbone monomer precursor and obtaining an intercalator-linker-backbone monomer precursor;

or

25 a2) providing a backbone monomer precursor unit comprising at least two reactive groups, said two reactive groups may optionally be individually protected and/or masked) and optionally comprising a linker part; and

b2) providing a linker precursor molecule comprising at least two reactive groups, said two reactive groups may optionally be individually protected; and

30 c2) reacting said monomer precursor unit with said linker precursor and thereby obtaining a backbone-linker; and

35 d2) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and optionally a linker part coupled to a reactive group; and

bases of a nucleic acid and optionally a linker part coupled to a reactive group;  
and

5 e2) reacting said intercalator with said backbone-linker and obtaining an intercalator-linker-backbone monomer precursor;

or

10 a3) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and a linker part coupled to a reactive group; and

b3) providing a backbone monomer precursor unit comprising at least two reactive groups, said two reactive groups may optionally be individually protected and/or masked), and a linker part; and  
15

c3) reacting said intercalator-linker part with said backbone monomer precursor-linker and obtaining an intercalator-linker-backbone monomer precursor;

20 and

f) optionally protecting and/ or de-protecting said intercalator-linker-backbone monomer precursor; and

25 g) providing a phosphor containing compound capable of linking two pseudonucleotides, nucleotides and/ or nucleotide analogues together; and

h) reacting said phosphorous containing compound with said intercalator-linker-backbone monomer precursor; and

30

i) obtaining an intercalator pseudonucleotide

Preferably, the intercalator reactive group is selected so that it may react with the linker reactive group. Hence, if the linker reactive group is a nucleophile, then  
35 preferably the intercalator reactive group is an electrophile, more preferably an

electrophile selected from the group consisting of halo alkyl, mesyloxy alkyl and tosyloxy alkyl. More preferably the intercalator reactive group is chloromethyl. Alternatively, the intercalator reactive group may be a nucleophile group for example a nucleophile group comprising hydroxy, thiol, selam, amine or mixture thereof.

5

Preferably, the cyclic or non cyclic alkane may be a polysubstituted alkane or alkoxy comprising at least three linker reactive groups. More preferably the polysubstituted alkane may comprise three nucleophilic groups such as, but not limited to, an alkane triole, an aminoalkan diol or mercaptoalkane diol. Preferably the polysubstituted

10 alkane contain one nucleophilic group that is more reactive than the others, alternatively two of the nucleophilic groups may be protected by a protecting group. More preferably the cyclic or non cyclic alkane is 2,2-dimethyl-4-methylhydroxy-1,3-dioxalan, even more preferably the alkane is D- $\alpha,\beta$ -isopropylidene glycerol .

15

Preferably, the linker reactive groups should be able to react with the intercalator reactive groups, for example the linker reactive groups may be a nucleophile group for example selected from the group consisting of hydroxy, thiol, selam and amine, preferably a hydroxy group. Alternatively the linker reactive group may be an electrophile group, for example selected from the group consisting of halogen,

20 triflates, mesylates and tosylates. In a preferred embodiment at least 2 linker reactive groups may be protected by a protecting group.

25

The method may furthermore comprise a step of attaching a protecting group to one or more reactive groups of the intercalator-precursor monomer. For example a DMT group may be added by providing a DMT coupled to a halogen, such as Cl, and reacting the DMT-Cl with at least one linker reactive group. Accordingly, preferably at least one linker reactive group will be available and one protected. If this step is done prior to reaction with the phosphor comprising agent, then the phosphor comprising agent may only interact with one linker reactive group.

30

The phosphor comprising agent may for example be a phosphoramidite, for example  $\text{NC}(\text{CH}_2)_2\text{OP}(\text{Npr}^1)_2$  or  $\text{NC}(\text{CH}_2)_2\text{OP}(\text{Npr}^1)_2\text{Cl}$ . Preferably the phosphor comprising agent may be reacted with the intercalator-precursor in the presence of a base, such as  $\text{N}(\text{et})_3$ ,  $\text{N}(\text{'pr})_2\text{Et}$  and  $\text{CH}_2\text{Cl}_2$ .

35

One specific non-limiting example of a method of synthesising an intercalator pseudonucleotide according to the present invention is outlined in example 1 and in figure 1.

5 Once the appropriate sequences of oligonucleotide or oligonucleotide analogue are determined, they are preferably chemically synthesised using commercially available methods and equipment: For example, the solid phase phosphoramidite method can be used to produce short oligonucleotide or oligonucleotide analogue comprising intercalator pseudonucleotides.

10

For example the oligonucleotides or oligonucleotide analogues may be synthesised by any of the methods described in "Current Protocols in Nucleic acid Chemistry" Volume 1, Beaucage et al., Wiley.

15

It is one objective of the present invention to provide methods of synthesising oligonucleotides or oligonucleotide analogues comprising at least one intercalator pseudonucleotide, wherein synthesis may comprise the steps of

20

a) bringing an intercalator pseudonucleotide according to the present invention into contact with a growing chain of a support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and

**b)** reacting said intercalator pseudonucleotide with said support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and

**c)** optionally capping unreacted said support-bound oligonucleotide; and

25

**d)** optionally further elongating said oligonucleotide analogue by adding one or more nucleotides, nucleotide analogues or intercalator pseudonucleotides to the oligonucleotide analogue in a desired sequence; and

**e)** cleaving said oligonucleotide analogue from said solid support; and

30

**f)** thereby obtaining said oligonucleotide analogue comprising at least one intercalator pseudonucleotide.

In one embodiment of the present invention the synthesis may comprise the steps of

35

a. bringing an intercalator pseudonucleotide according to the invention comprising a reactive group, which may be protected by an acid la-

- bile protection group into contact with a growing chain of a support-bound oligonucleotide or oligonucleotide analogue; and
- b. reacting said intercalator pseudonucleotide with said support-bound oligonucleotide or oligonucleotide analogue; and
- 5 c. washing away excess reactants from product on the support; and
- d. optionally capping unreacted said support-bound oligonucleotide; and
- e. oxidizing the phosphite product to phosphate product; and
- f. washing away excess reactants from product on support; and
- 10 g. optionally capping unreacted said support-bound oligonucleotide; and
- h. repeating steps a)-g) until the desired number of intercalator pseudonucleotides are inserted; and
- i. optionally elongating said support-bound oligonucleotide containing at least one intercalator pseudonucleotide; and
- j. optionally repeating step a-i)
- 15 k. cleaving oligonucleotide analogue from solid support and removing base labile protecting groups in basic media; and
- l. purifying oligonucleotide analogue containing acid labile protecting group; and
- m. removing acid labile protecting group with acidic media; and
- 20 n. obtaining a terminus pseudonucleotide modified oligonucleotide analogue containing at least one intercalator pseudonucleotide

In another embodiment of the present invention the synthesis may comprise the steps of

- 25 a) bringing an intercalator pseudonucleotide according to the present invention into contact with an universal support; and
- b) reacting said intercalator pseudonucleotide with the universal support; followed by step c) to j) as described in the method herein above.

30

It is also contained within the present invention that the last acid labile protection group may be removed prior to cleavage of the support-bound oligonucleotide analogue. Subsequent purification of the oligonucleotide analogue is optional.

In yet another embodiment of the present invention the method comprises the synthesis an oligonucleotide or oligonucleotide analogue comprising at least one internally positioned intercalator pseudonucleotide, wherein synthesis may comprise the steps of

5

- a) bringing a nucleotide or nucleotide analogue protected with an acid labile protection group into contact with a growing chain of a support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
- 10 b) reacting the protected nucleotide analogue with the growing chain of said support-bound nucleotide, oligonucleotide, nucleotide analogue or oligonucleotide analogue; and
- c) washing away excess reactants from product on support; and
- d) optionally capping unreacted said support-bound nucleotide; and
- 15 e) oxidizing the phosphite product to phosphate product; and
- f) washing away excess reactants from product on support; and
- g) optionally capping unreacted said support-bound nucleotide; and
- h) removing acid labile protecting group; and
- i) washing away excess reactants from product on the support; and
- 20 j) repeating steps a)-f) to obtain the desired oligonucleotide analogue sequence; and
- k) cleaving the oligonucleotide analogue from solid support and removing base labile protecting groups in basic media; and
- l) purifying oligonucleotide containing acid labile protecting group; and
- 25 m) removing acid labile protecting group; and
- n) obtaining an intercalator modified oligonucleotide analogue.

30

Alternatively the last acid labile protection group may be removed prior to cleavage of the support-bound oligonucleotide analogue. Purification of the oligonucleotide analogue is optional.

### Template

35

The term "template" according to the present invention refers to a nucleic acid or nucleic acid analogue. Preferably a template comprises a nucleotide sequence that

corresponds to a specific oligonucleotide or oligonucleotide analogue. If said sequence comprised within said template is internally positioned, hybridization between said template and said oligonucleotide or oligonucleotide analogue will create a 3' free end of said oligonucleotide or oligonucleotide analogue. This 3' free end is capable of being extended by a for example a DNA polymerase in a template directed manner and said oligonucleotide or oligonucleotide analogue is thus said to prime extension. The term "primer" therefore covers a oligonucleotide or oligonucleotide analogue that is able to prime template directed extension by DNA and RNA polymerases.

#### Linear oligonucleotides comprising intercalator pseudonucleotides

In one aspect the present invention relates to an oligonucleotide and/or oligonucleotide analogue comprising at least one intercalator pseudonucleotide of the general structure



wherein

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/ or intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides; and

wherein the second sequence is capable of hybridizing to a homologously complementary target sequence comprised within a nucleic acid or nucleic acid analogue and is capable of priming a template directed extension reaction.

5     The at least one intercalator pseudonucleotide may be inserted at any desired position within the first and/ or second sequence. If the oligonucleotide analogue comprises more than one intercalator pseudonucleotides, preferably every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue.

10

Accordingly, the intercalator pseudonucleotide may be any of the intercalator pseudonucleotides described herein above.

15

In one embodiment, the first sequence is attached as a tail sequence to the second sequence and meant to be rather inactive in sequence specific hybridisation. In this embodiment, the second sequence alone is preferentially capable of hybridizing to a target nucleic acid or nucleic acid analogue, and the first sequence preferentially contains neighboring intercalator pseudonucleotides according to the present invention.

20

The first sequence preferably consists of  $n$  nucleotides and/or nucleotide analogues and intercalator pseudonucleotides, wherein  $n$  may be any suitable integer.

25

Preferably,  $n$  is an integer in the range from 1 to 150, such as from 1 to 50, such as from 5 to 50, such as from 10 to 20, for example  $n$  is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

30

The second sequence preferably consists of  $m$  nucleotides and/or nucleotide analogues and/ or intercalator pseudonucleotides, wherein  $m$  may be any suitable integer. Preferably,  $m$  is an integer in the range from 1 to 150, such as from 1 to 50, such as from 5 to 50, such as from 10 to 20, for example  $n$  is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

35



If the oligonucleotide analogue comprises more than one intercalator pseudonucleotides, preferably every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue.

- 5 In one preferred embodiment  $n$  is smaller than  $m$ . However it is also contained within the present invention that  $m$  is equal to  $n$ .

The first and second sequence of the oligonucleotide analogues according to the present invention may comprise any desirable number of intercalator  
10 pseudonucleotides. For example the oligonucleotide or oligonucleotide analogue may comprise from 2 to 20, such as from 2 to 10, such as from 2 to 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

- 15 In another embodiment, said first sequence is not merely a tail attached to said second sequence.

The intercalator pseudonucleotides may be placed in any desirable position within the first and the second sequence. For example, an intercalator pseudonucleotide  
20 may be placed at either end of the first and second sequence or an intercalator pseudonucleotide may be placed in an internal position within the first and second sequence. For example, the first sequence comprises at least two closely positioned intercalator pseudonucleotides.

- 25 Furthermore, if the oligonucleotide analogue comprises more than one intercalator pseudonucleotide, said pseudonucleotides may be placed in relation to each other in any desirable manner. For example, they may be placed so that 0, such as 1, for example 2, such as 3, for example 4, such as 5, for example from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as more than 20 nucleotides and/ or  
30 nucleotide analogues are separating the intercalator pseudonucleotides. In a preferred embodiment, the oligonucleotide analogue comprises at least one pair of intercalator pseudonucleotides capable of forming an intramolecular excimer, intramolecular exciplex, a FRET complex and/or a charge-transfer complex.

In a preferred embodiment, the first sequence is positioned upstream of said second sequence. It is furthermore preferred that said first sequence comprises at least one pair of intercalator pseudonucleotides capable of forming an intramolecular excimer, intramolecular exciplex, a FRET complex and/or a charge-transfer complex. In a more preferred embodiment an oligonucleotide analogue comprises said first and second sequence, where said second sequence comprises only one intercalator pseudonucleotide positioned in the 5' end and where said second sequence is able to hybridize to a homologously complementary target sequence and prime template directed extension.

The first and second sequence of the oligonucleotide analogue may comprise more than one intercalator pseudonucleotide, wherein said intercalator pseudonucleotides may be similar or may be different. If the oligonucleotide analogue comprises more than one intercalator pseudonucleotides, preferably every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue.

The oligonucleotide analogues may comprise nucleotides comprised within DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA and HNA. Accordingly, the oligonucleotide analogue may comprise one or more selected from the group consisting of subunits of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA and HNA, i.e. the oligonucleotide analogue may be selected from the group of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA, HNA and mixtures thereof.

In a preferred embodiment only the second sequence of an oligonucleotide analogue comprising a first and a second sequence according to the present invention is capable of hybridizing to a homologously complementary target sequence under high-stringency conditions. In a more preferred embodiment, said second sequence of said oligonucleotide analogue is only capable of hybridizing to a complementary target sequence under high-stringency conditions.

#### Hairpin oligonucleotides comprising intercalator pseudonucleotides

In one aspect the present invention relates to an oligonucleotide analogue comprising at least one intercalator pseudonucleotide of the general structure

X-Y-Q

wherein

5

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

10

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

15

wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/ or intercalator pseudonucleotides, wherein said first sequence is capable of hybridizing to said second sequence.

20

Accordingly, the first sequence constitutes a sequence part of the oligonucleotide analogue and the second sequence constitutes another sequence part of the oligonucleotide analogue.

25

The first and the second sequence may be separated by a third consisting of p nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. Preferably, the second and third sequence are capable of hybridizing to a homologously complementary target sequence comprised within a nucleic acid or nucleic acid analogue and are capable of priming a template directed extension reaction.

30

The at least one intercalator pseudonucleotide may be inserted at any desired position within the first, second and/or third sequence. Preferably, if more than one intercalator pseudonucleotides are present, every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue.

35

The intercalator pseudonucleotide may be any of the intercalator pseudonucleotides described herein above.

5       The first sequence preferably consists of  $n$  nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein  $n$  may be any suitable integer. Preferably,  $n$  is an integer in the range from 5 to 50, for example  $n$  is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.

10

The second sequence preferably consists of  $m$  nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein  $m$  may be any suitable integer. Preferably,  $m$  is an integer in the range from 5 to 50, for example  $n$  is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, 15       such as from 20 to 30, for example from 30 to 50.

20

In one preferred embodiment the first sequence may hybridize with the second sequence essentially over the entire length of the first sequence and the second sequence, even more preferably nucleotides and nucleotide analogues in the duplex are essentially complementary.

25

The oligonucleotide analogues according to the present invention should as a minimum be as long as the first sequence and the second sequence together, however the oligonucleotide analogues may be longer than the first sequence and the second sequence together, and accordingly the oligonucleotide analogues may 25       comprise other parts than the first sequence and the second sequence.

30

For example, the oligonucleotide analogue preferably comprises between 5 and 100, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20, such as between 20 and 30, for example between 30 and 40, such as between 40 and 50, for example between 50 and 60, such as between 60 and 80, for example between 60 and 100 nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides. More preferably the oligonucleotide analogue consists of in the range from 15 to 50 nucleotides.

35

In another embodiment, an oligonucleotide analogue according to the invention may comprise a first sequence and a second sequence, wherein said first sequence and said second sequence are separated by a third sequence consisting of p nucleotides and/or nucleotide analogues.

5

p may be any desirable integer, for example p may be an integer between 1 and 5, for example between 5 and 10, such as between 10 and 15, for example between 15 and 20, such as between 20 and 30, for example between 30 and 50.

10

The oligonucleotide analogues according to the present invention may comprise any desirable number of intercalator pseudonucleotides. For example the oligonucleotide analogue may comprise between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

15

The intercalator pseudonucleotides may be positioned at any position within the first sequence of the oligonucleotide analogue.

20

Accordingly, the present invention relates to oligonucleotide analogues according to the present invention, wherein the first sequence comprises at least one, for example between 2 and 5, such as between 5 and 10, such as between 10 and 15, for example between 15 and 20 intercalator pseudonucleotides.

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Furthermore, the invention relates to oligonucleotide and/or oligonucleotide analogues according to the present invention, wherein the second sequence comprises no intercalator pseudonucleotides.

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The intercalator pseudonucleotides may be placed in any desirable position within the first sequence of the oligonucleotide analogues. For example, an intercalator pseudonucleotide may be placed at either end of the first sequence of the oligonucleotide analogues or an intercalator pseudonucleotide may be placed in an internal position within the oligonucleotide analogues.

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Furthermore, if the oligonucleotide analogue comprises more than one intercalator pseudonucleotides, they may be placed in relation to each other in any desirable

manner. For example, they may be placed so that 1, for example 2, such as 3, for example 4, such as 5, for example from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as more than 20 nucleotides are separating the intercalator pseudonucleotides. In a preferred embodiment at least two intercalator pseudonucleotides are placed so the intercalators of said intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex, a intramolecular FRET complex and/or a intramolecular charge-transfer complex.

- 10 The oligonucleotide analogue may comprise more than one intercalator pseudonucleotide, wherein said intercalator pseudonucleotides may be similar or may be different.

The oligonucleotide analogues may comprise any nucleotides or nucleotide analogues. Nucleotides and nucleotide analogues can for example be chosen from the group consisting of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA and HNA. Accordingly, the oligonucleotide analogue may comprise one or more selected from the group consisting of subunits of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA and HNA, i.e. the oligonucleotide analogue may be selected from the group of DNA, RNA, LNA, PNA, ANA, MNA, 2'-O-methyl RNA, HNA and mixtures thereof.

In one preferred embodiment an oligonucleotide analogue comprises a first and a second sequence, where said first sequence is positioned upstream of said second sequence and where said second sequence is able to hybridize to a homologously complementary target sequence and prime template directed extension.

Said second and third sequence may either hybridise to said target sequence as one consecutive sequence, thus said second sequence is positioned downstream of said third sequence. Alternatively, said third sequence may fold back and hybridise to the elongated second sequence and thus hybridise downstream of said second sequence. This last alternative is referred to as a Scorpion mechanism (Whitcombe et al. 1999, Nature Biotechnology 17: 804-807).

In one embodiment of the present invention it is preferred that the melting temperature of a hybrid of the first sequence and the second sequence is

significantly lower than the melting temperature of a hybrid between the second sequence and a homologously complementary, preferably complementary, target nucleic acid or nucleic acid analogue.

5 In another preferred embodiment an oligonucleotide analogue comprises a first, a second and a third sequence, where said first sequence is positioned upstream of said second and third sequence and where said second and third sequence are able to hybridize to a homologously complementary target sequence and prime template directed extension.

10

In one embodiment of the present invention it is preferred that the melting temperature of a hybrid of the first sequence and the second sequence is significantly lower than the melting temperature of a hybrid between the second sequence and a homologously complementary target nucleic acid or nucleic acid analogue.

15

In one preferred embodiment the melting temperature of a hybrid of the first and second sequence is significantly higher than the melting temperature of a hybrid between the first sequence and a non-corresponding nucleic acid or nucleic acid analogue.

20

In another preferred embodiment the melting temperature of a hybrid of the first and second sequence is significantly higher than the melting temperature of a hybrid between the second sequence and a non-corresponding nucleic acid or nucleic acid analogue.

25

In yet another preferred embodiment of the present invention the melting temperature of a hybrid of the first sequence and the second sequence is significantly lower than the melting temperature of a hybrid between the second and third sequence and a homologously complementary nucleic acid or nucleic acid analogue. Furthermore, the melting temperature of a hybrid of the first sequence and the second sequence may be significantly lower than the melting temperature of a hybrid between the second and a homologously complementary nucleic acid or nucleic acid analogue.

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In a more preferred embodiment of the present invention the melting temperature of a hybrid of the first and second sequence is significantly higher than the melting temperature of a hybrid between the second and third sequence and a non-complementary nucleic acid or nucleic acid analogue.

5

Preferably the homologously complementary target nucleic acid or nucleic acid analogue is DNA or a nucleic acid analogue derived from a DNA template by extending oligonucleotides according to the present invention.

10

When the melting temperature of a hybrid of the first sequence and the second sequence is significantly lower than the melting temperature of a hybrid between the second and optionally third sequence and a homologously complementary nucleic acid or nucleic acid analogue, for example DNA, that results in the advantageous effect that in a mixture comprising the oligonucleotide analogue comprising the first sequence and the second sequence and said nucleic acid or nucleic acid analogue homologously complementary to the second and optionally third sequence, the second and optionally third sequence will preferably hybridize with said homologously complementary nucleic acid or nucleic acid analogue, rather than with the first sequence.

20

Accordingly, in a mixture comprising an oligonucleotide analogue according to the present invention, if the first sequence and the second sequence are hybridized, that is indicative of that only a limiting amount or for example no homologously complementary target nucleic acids and/ or nucleic acid analogues are available.

25

Vice versa, in a mixture comprising the oligonucleotide and/or oligonucleotide analogues, if the first sequence and the second sequence are not hybridized, that is indicative of that the mixture furthermore comprises homologously complementary nucleic acids and/ or nucleic acid analogues.

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Furthermore, if a limited amount of said homologously complementary target nucleic acids and/ or nucleic acid analogues is available, an amount of said oligonucleotide analogues corresponding to the amount of said homologously complementary targets will be hybridized to said homologously complementary targets and the



remaining amount of said oligonucleotide analogue first sequences will be hybridized to said second sequences.

5 One non-limiting example of an hairpin oligonucleotide analogue according to the invention is given in figure 5.

10 In another preferred embodiment an oligonucleotide or oligonucleotide analogue comprising a first, a second and optionally a third sequence according to the present invention is affixed to a solid surface.

In a more preferred embodiment an array of oligonucleotides or oligonucleotide analogues comprising a first, a second and optionally a third sequence according to the present invention is affixed to a solid surface.

#### Excimer, exciplex, FRET and charge transfer

15 An excimer is a dimer of compounds, which is associated in an electronic excited state, and which is dissociative in its ground state. When an isolated compound is excited it may lose its excitation or it may associate with another compound of the same kind (which is not excited), whereby an excimer is formed. An excimer emits  
20 fluorescence at a wavelength different from monomer fluorescence emission. When the excimer loses its excitation the association is no longer favourable and the two species will dissociate. An exciplex is an excimer like dimer, wherein the two compounds are different.

25 Intramolecular excimers are formed by two moieties comprised within one molecule, for example 2 polyaromatic groups within the same molecule. Similar intramolecular exciplexes are formed by two moieties comprised within one molecule, for example by 2 different polyaromatic groups.

30 Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. FRET is dependent on the inverse 6<sup>th</sup> power of the intermolecular distance, making it useful over distances comparable with the dimension of biological

macromolecules. Preferably the donor and the acceptor must be in close proximity (typically between 10 to 100 Å) for FRET to occur. Furthermore, the absorption spectrum of the acceptor must overlap with the fluorescence emission spectrum of the donor. It is further preferred that the donor and the acceptor transition dipole orientations must be approximately parallel.

A charge transfer complex is a chemical complex in which there is weak coordination involving the transfer of charge between two intermolecular or intramolecular moieties, called an electron donor and an electron acceptor. These two moieties exhibit an observable charge-transfer absorption band during charge-transfer transition. An example is phenoxinone, in which the phenol and quinone molecules are not held together by formal chemical bonds but are associated by transfer of charge between the compounds' aromatic ring systems.

#### Spectral properties

Spectral properties, such as fluorescence, are preferred for detection of hybridisation, for example when carrying out real-time detection of for example quantitative PCR processes. Thus, fluorescent labels compatible with association to nucleic acids and nucleic acid analogues are needed.

In one preferred embodiment of the present invention, intercalator pseudonucleotides comprise fluorescence properties that may be used for detection of hybridisation and/or extension.

In another preferred embodiment monomer fluorescence of intercalator pseudonucleotides may be used to detect hybridisation or to detect the presence of extension products or to follow the real-time formation of extension products.

In a more preferred embodiment of the present invention the oligonucleotide analogue comprises a first sequence, which comprises at least two intercalator pseudonucleotides and said intercalator pseudonucleotides are capable of forming an excimer and/ or an exciplex and/ or a charge-transfer and/ or a FRET complex.

Preferably said two intercalators of an oligonucleotide analogue, is placed in a

distance from one another within the oligonucleotide analogue so they can interact and hence form an intramolecular excimer, an intramolecular exciplex, a FRET complex or a charge transfer complex.

5 In another preferred embodiment of the present invention the intercalators of at least two intercalator pseudonucleotides within said first sequence are capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex and/ or an intramolecular charge transfer complex, when at least one of the k nucleotides at either side of any of said intercalator pseudonucleotides are  
10 unhybridised.

More preferably the intercalators of at least two intercalator pseudonucleotides within said first sequence are capable of forming an intramolecular excimer and/ or an intramolecular exciplex and/ or an intramolecular FRET complex and/ or an  
15 intramolecular charge transfer complex, when at least one of the basepairs comprised within the k nucleotides at either side of any of said intercalator pseudonucleotides is unhybridised and said intercalators are not capable of forming an intramolecular excimer, an intramolecular exciplex, an intramolecular FRET complex or an intramolecular charge transfer complex, when all the k nucleotides at  
20 either side of any of said intercalator pseudonucleotides are hybridized.

Above, k is an integer in the range of 1 to 3.

25 In another preferred embodiment of the present invention the intercalators of said first sequence are capable of forming an intermolecular excimer, an intermolecular exciplex, an intermolecular FRET complex and/ or an intermolecular charge transfer complex, when the second sequence of the oligonucleotide analogue comprising intercalator pseudonucleotides is not hybridized to the first sequence.

30 It is only possible for two molecular moieties to form an intramolecular excimer, an intramolecular exciplex, FRET or a charge transfer complex if the two molecules are positioned in relation to one another, so that they are able to interact with one another. Hence intercalators cannot form an intramolecular excimer, an intramolecular exciplex, FRET complex or a charge transfer complex if there is a  
35 molecular moiety separating the intercalators.

Intercalators according to the present invention are capable of co-stacking with nucleobases. When oligonucleotide analogues comprising said intercalators hybridize with homologously complementary DNA, said intercalators will preferably co-stack with the nucleobases of the hybrid. If all  $k$  nucleobases,  $k$  being an integer in the range of 1 to 3, around each of the at least two intercalators form matched base-pairs, this will preferably result in a steric hindrance of the intercalator moieties, so that said intercalators will not be able to interact and accordingly not be able to form an intramolecular excimer, an intramolecular exciplex, FRET complex or a charge transfer complex.

Accordingly, preferred oligonucleotide and/or oligonucleotide analogue according to the present invention are oligonucleotide and/or oligonucleotide analogues, wherein the first sequence comprises at least two intercalator pseudonucleotides, wherein the intercalators of said intercalator pseudonucleotides are capable of forming an intramolecular excimer, an intramolecular exciplex or a FRET complex or a charge-transfer complex, when the first sequence is not hybridized with the second sequence or another nucleic acid.

Such intercalators employed according to the present invention for example include, but is not limited to, pyrene.

The oligonucleotide analogue according to the present invention may in one embodiment comprise one or more directly or indirectly detectable labels in addition to intercalator pseudonucleotides.

In one embodiment, the oligonucleotide analogue according to the present invention may comprise fluorescent groups in addition to intercalator pseudonucleotides. The fluorescent groups may be useful for detecting the oligonucleotide analogue.

The fluorescent group may be any group capable of fluorescing, in particular any group capable of fluorescing when it is associated with said first or second sequence of the oligonucleotide analogue according to the present invention and/or when it is incorporated into a nucleic acid or nucleic acid analogue. For example the fluorescent group may be selected from the group consisting of fluorescein, FITC,

rhodamine, lissamine rhodamine, rhodamine 123, coumarin, CY-2, CY-3, CY 3.5, CY-5, CY 5.5, FAM, GFP, YFP, BFP, YO-YO, HEX, JOE, Nano Orange, Nile Red, OliGreen, Oregon Green, Pico green, Radiant Red, Ribo Green, ROX, R-phycoerythrin, SYPRO Orange, SYPRO Red, SYPRO Ruby, TAMRA, Texas Red, XRITC, Propidium iodide, Acridine Orange, ethidium bromide, SYBR Gold, SYBR Green I, and SYBR Green II..

Furthermore, it is also contained within the present invention that the oligonucleotide analogue may comprise at least one quencher molecule. A quencher molecule according to the present invention is any molecule that is capable of quenching the fluorescence of a fluorescent group in its vicinity. The quencher may function by absorbing energy from the fluorescent group and dissipating the energy as heat or radiative decay. Hence, the signal from the fluorescent group will be reduced or absent. Accordingly, if a fluorescent group and a suitable quencher molecule are placed close to each other, the fluorescence of the fluorescent group will be quenched.

Examples of quencher molecules include, but are not limited to, DABCYL, DABSYL, TAMRA, Methyl red, Black Hole-1, Black Hole-2, ElleQuencher and QSY-7. However, the quencher molecule must generally be selected according to the fluorescent group.

Preferred pairs of fluorescent group-quencher molecules according to the present invention includes, but is not limited to:

<u>Fluorescent group</u>	<u>Quencher</u>
FAM	TAMRA
TET	TAMRA
Rhodamine	TAMRA
Coumarin	DABCYL
EDANS	DABCYL
Fluorescein	DABCYL
Lucifer Yellow	DABCYL
Eosin	DABCYL
TAMRA	DABCYL

In particular, the oligonucleotide and/or oligonucleotide analogue according to the present invention may comprise both a fluorescent group and a quencher molecule. The fluorescent group may be placed as dangling end in the 5' end or in the 3' end or both ends. It is also preferred that at least one quencher molecule is attached as a dangling end, more preferably all non-intercalator pseudonucleotide quencher molecules are attached as dangling ends. The quencher molecule may be placed as a dangling end in the 5' end or in the 3' end or both ends. Preferably, one the fluorescent group or one quencher molecule are placed as a dangling end, in the 5' end of the oligonucleotide and/or oligonucleotide analogue. Further, if one fluorescent group is placed as a dangling end in the 5' end, preferably a quencher is positioned internally. Vice versa, if one quencher molecule is placed as a dangling end in the 5' end of the oligonucleotide and/or oligonucleotide analogue, one fluorescent group is positioned internally.

Accordingly, the fluorescent group and the quencher molecule are positioned in relation to each other so that the quencher molecule is capable of quenching the fluorescence or part of the fluorescence of the fluorescent group when the first sequence is hybridized to the second sequence, but is not capable of quenching the fluorescence of the fluorescent group when the first sequence is not hybridized to the second sequence.

In an even more preferred embodiment an oligonucleotide and/or oligonucleotide analogue comprise a first sequence and a homologously complementary second sequence that are separated by a third sequence (a hairpin probe), where at least one of the sequences comprise at least one intercalator pseudonucleotide and the oligonucleotide and/or oligonucleotide analogue has an additional fluorescent group placed as dangling end in the 5' end, and quencher molecule internally positioned. Vice versa, if one quencher molecule is placed as a dangling end in the 5' end of the oligonucleotide and/or oligonucleotide analogue, one fluorescent group is positioned internally.

It is preferred that the quencher molecule is capable of quenching the fluorescence or part of the fluorescence of the fluorescent group when the first sequence is hybridized to the second sequence, but is not capable of quenching the

fluorescence of the fluorescent group when the first sequence is not hybridized to the second sequence. In the most preferred embodiment, this is obtained when said quencher molecule and fluorescent group come into close proximity when the first and second sequences of said oligonucleotide and/or oligonucleotide analogue hybridize.

Accordingly it is a preferred embodiment of the present invention to provide a hairpin probe that will self-hybridize unless subjected to a fully complementary target under hybridization conditions.

When the label is a complex of at least two intercalator pseudonucleotides capable of forming an intramolecular excimer, exciplex, FRET or charge-transfer complex (see herein below) with the proviso that every two intercalator pseudonucleotides are separated by at least one nucleotide or nucleotide analogue, quenching of signal could be obtained by hybridization of the nucleotides in a region of at least one nucleotide to either side of any intercalator pseudonucleotide in the complex to a complementary sequence.

Hence it is a preferred embodiment of the present invention to provide an oligonucleotide and/or oligonucleotide analogue comprising at least two intercalator pseudonucleotides where the spectral properties are changed upon hybridization to a target nucleic acid or as a consequence of amplification of a target nucleic acid. In a preferred embodiment the spectral signal is low when there is no or small amounts of a target nucleic acids, and high when there is larger amounts of target nucleic acid present. When used under an amplification reaction of target sequence, e.g. by PCR, it is preferred that the spectral signal increases in correspondence to the increase of said target nucleic acid sequence.

Accordingly it is preferred to provide an oligonucleotide and/or oligonucleotide analogue that comprise a first sequence and a complementary second sequence that are separated by a third sequence (a hairpin probe), where second or third sequence comprise at least one intercalator pseudonucleotide and where first sequence comprise an additional complex of intercalator pseudonucleotides according to the present invention, where the spectral signal is low when said first sequence is hybridized to second sequence and high when they are not hybridized.

It is also comprised within the present invention that the first sequence may comprise a donor for FRET and the second sequence may comprise an acceptor for a FRET or vice versa. Preferably said donor and said acceptor are positioned so that FRET may occur when the first sequence is hybridized to the second sequence and accordingly FRET fluorescence may only be detected when the first sequence is hybridized to the second sequence.

FRET donor and acceptor pairs for example includes:

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<u>Donor</u>	<u>Acceptor</u>
Fluorescein	Tetramethylrhodamine
IAEDANS	fluorescein
EDANS	DABCYL
Fluorescein	Fluorescein
BODIPY FL	BODIPY FL
Fluorescein	QSY 7 dye

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Furthermore, hybridisation or the formation of nucleotide or nucleotide analogue extension products constituting double stranded nucleic acids and/ or nucleic acid analogues, e.g. DNA, may also be detected using a label not associated directly with the oligonucleotide and/or oligonucleotide analogues and optionally oligonucleotides used for priming the extension. In one embodiment the oligonucleotide and/or oligonucleotide analogues of the present invention are used to prime template directed extension, where after a label specific for double stranded nucleic acids and/ or nucleic acid derivatives is used to detect extension products.

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In one embodiment such a label may be selected from the group consisting of, but not limited to, Propidium iodide, Acridine Orange, ethidium bromide, SYBR Gold, SYBR Green I, and SYBR Green II. The fluorescence properties of a fluorescent group may be modulated according to buffer conditions and physical factors. For instance temperature, ionic strength and pH are parameters that can affect the strength of the fluorescent signal obtained from a specific fluorophore. An example of a different chemical group of substances that are also known to affect fluores-



cence of pyrene excimers is the lipophilic surfactant molecules, as disclosed in US patent 5,466,578. The authors teach that the cationic subgroup of surfactants comprising a quarternary ammonium salt with carbon chains attached, like e.g. hexadecyltrimethyl ammonium bromide, can be used to significantly increase the signal from oligonucleotides end-labeled with pyrene excimers.

It is an embodiment of the present invention to perform fluorescence detection in the presence of surfactants to significantly increase the fluorescence of fluorescent groups of an oligonucleotide or oligonucleotide analogue, when said oligonucleotide or oligonucleotide analogue comprises intercalator pseudonucleotides according to the present invention.

It is a further embodiment of the present invention to perform fluorescence detection of fluorescence of intercalator pseudonucleotides according to the present invention in the presence of surfactants to significantly increase the fluorescence of said intercalator pseudonucleotides, when said intercalator pseudonucleotides are comprised within an oligo nucleotide or oligo nucleotide analogue.

It is a preferred embodiment of the present invention to perform fluorescence detection of excimer, exciplex, FRET or charge-transfer fluorescence of intercalator pseudonucleotides according to the present invention in the presence of surfactants, to significantly increase the excimer, exciplex, FRET or charge-transfer fluorescence of said intercalator pseudonucleotides, when said intercalator pseudonucleotides are comprised within an oligo nucleotide or oligo nucleotide analogue.

It is a further preferred embodiment of the present invention to perform fluorescence detection of excimer, exciplex, FRET or charge-transfer fluorescence of intercalator pseudonucleotides according to the present invention, when said intercalator pseudonucleotides are positioned internally in an oligo nucleotide or oligo nucleotide analogue, in the presence of surfactants to significantly increase the excimer, exciplex, FRET or charge-transfer fluorescence of said intercalator pseudonucleotides.

It is a most preferred embodiment of the present invention to perform fluorescence detection of excimer, exciplex, FRET or charge-transfer fluorescence of intercalator pseudonucleotides, containing only one pyrene moiety, according to the present

invention, when said intercalator pseudonucleotides are positioned internally in an oligo nucleotide or oligo nucleotide analogue, in the presence of surfactants to significantly increase the excimer, exciplex, FRET or charge-transfer fluorescence of said intercalator pseudonucleotides.

5

Since the positive effect of surfactants on the fluorescence strength of especially dimer (or higher order) complexes of pyrene may be due to surfactant molecule displacement of water molecules from the dimer, acting to quench fluorescence, other lipophilic molecules may have similar effects on fluorescence strength. Lipophilic groups and other molecules that could displace water from intercalator dimers are often used in standard assay buffers. Examples of such molecules that have been shown to affect fluorescence strength are Triton X-100, Sodium Dodecyl Sulphate, Glycerol and DMSO.

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#### 15     Additional labels

In addition to fluorescent labels, oligonucleotides, oligonucleotide analogues, nucleic acids or nucleic acid analogues may be labeled by other means. This may be a preferred option if the fluorescent properties of the oligonucleotide and/or oligonucleotide analogues according to the present invention or other additional fluorescent labels are not used for detection. An example is when exploiting only the abilities of said oligonucleotide and/or oligonucleotide analogues to reduce the formation of unwanted extension products.

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The most common methods of detection, not including oligonucleotide and/or oligonucleotide analogues with directly associated fluorescent labels, are the use of ligands that bind to labeled antibodies, the use of radioactive labels and the use of chemiluminescent agents.

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The choice label depends on the sensitivity, ease of conjugation with the oligonucleotide and/or oligonucleotide analogue, stability requirements, and available instrumentation.

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Non-radioactive probes are often labeled by indirect means. Labels include antibodies, which can serve as specific binding pair members for a labeled ligand. Generally, one or more ligand molecule(s) is/are covalently bound to the probe, in this

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case the oligonucleotide and/or oligonucleotide analogue. The ligand(s) then binds to an anti-ligand molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

5

Ligands and anti-ligands may be varied widely. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

10

Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, AMPPD ([3-(2'-spiroamantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxetane]) and 2,3-dihydrophthalazinediones, e.g., luminol.

15

Oligonucleotide analogues may also be labeled with  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{33}\text{P}$  or  $^{32}\text{P}$  and subsequently detected by autoradiography. Preferably a normal PCR including hot dNTPs is carried out, whereby only the extension product will be radioactively labeled.

20

The above-mentioned methods will typically require some sort of separation of extended and non-extended oligonucleotide and/or oligonucleotide analogues after extension of oligonucleotide and/or oligonucleotide analogues have been carried out, but prior to detection. The method of separation chosen will typically depend on the labeling method used and the availability of procedures and instruments. Examples of separation methods are described herein below.

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### 30 Special features of oligonucleotide and/or oligonucleotide analogues comprising intercalator pseudonucleotides

The high affinity of oligonucleotide and/or oligonucleotide analogues according to the present invention toward nucleic acids or nucleic acid analogues and the spectral properties are not the only special features possessed by this

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oligonucleotide and/or oligonucleotide analogue. In addition, several other features added by the incorporation of intercalator pseudonucleotide moieties may be exploited. The first strategy relates to the presence of intercalator pseudonucleotides, which can decrease hybridization strength of homologously complementary sequences, both comprising intercalator pseudonucleotides, thereby preventing cross-hybridization. The second strategy relates to the sterical hindrance posed by the intercalator pseudonucleotide, being a non-natural nucleotide, which might also inhibit a DNA or RNA polymerase of proceeding beyond an intercalator pseudonucleotide present in the template strand, enabling precise control of extension-termination. The third strategy relates to exploiting the properties of a hairpin structure to inhibit unwanted priming.

According to the first strategy, the present invention provides methods of reducing cross-hybridization between regions of individual oligonucleotide and/or oligonucleotide analogues comprising intercalator pseudonucleotides.

Primer-dimers are unwanted PCR extension products arising from cross-hybridization among oligonucleotides or oligonucleotide analogues used for primers in the PCR reaction. Likewise, extension products arising from cross-hybridization between other nucleic acids or nucleic acid analogues in the PCR reaction, e.g. hybridization probes, also lead to unwanted extension products. Thus, the formation of unwanted extension products is a phenomenon general to PCR methods, and in particular, it is a problem when performing PCR with many cycles. When a PCR product is intended for later use, a template extension product essentially free of DNA contaminants is often required. Therefore, the presence of unwanted extension products may impose the need for additional and time-consuming purification steps. Another example where the presence of unspecific products, especially primer-dimers, is a major concern is in quantitative PCR when using unspecific stains for detection of double stranded nucleic acids or nucleic acid analogues.

Since different intercalator pseudonucleotides may provide different degrees of sterical hindrance to a polymerase, different intercalator pseudonucleotides may be chosen according to their properties in hindering the polymerase of proceeding beyond an intercalating pseudonucleotide present in the template strand and chosen according to a particular interest.

The use of intercalator pseudonucleotides as PCR terminators does not exclude the use of other PCR terminators, also referred to as "PCR stoppers", known in the art.

- 5 In a preferred embodiment oligonucleotide and/or oligonucleotide analogues according to the present invention inhibit the formation of primer-dimers by inhibiting cross-hybridization among said oligonucleotide and/or oligonucleotide analogues when used as primers for template directed extension.
- 10 The use of oligonucleotide and/or oligonucleotide analogues according to the present invention may also reduce the formation of primer-dimers even if intercalator pseudonucleotides are not capable of reducing cross-hybridization between primers. This second strategy of reducing unwanted extension products exploits that
- 15 intercalator pseudonucleotides pose a sterical hindrance to polymerases as outlined below. Preferably, the first sequence of oligonucleotide and/or oligonucleotide analogues comprising intercalating pseudonucleotides as described herein above, are positioned in the upstream region of the oligonucleotide and/or oligonucleotide analogue. Another complementary, homologously complementary or non-
- 20 corresponding oligonucleotide and/or oligonucleotide analogue designed the same way may be capable of hybridizing to the first mentioned oligonucleotide and/or oligonucleotide analogue. Upon hybridization intercalator pseudonucleotides present in the oligonucleotide and/or oligonucleotide analogue, serving as template for another oligonucleotide and/or oligonucleotide analogue priming extension, will be
- 25 positioned downstream of the extension site. Since nucleic acids are extended only in the 5' to 3' direction, intercalator pseudonucleotides of said oligonucleotide and/or oligonucleotide analogue template will inhibit a DNA or RNA polymerase in proceeding beyond said intercalator pseudonucleotides. Therefore, only a limited number of nucleotides or nucleotide analogues will be added.
- 30 Accordingly, in one embodiment the use of oligonucleotide and/or oligonucleotide analogues comprising intercalator pseudonucleotides according to the present invention reduces the formation of unwanted extension products in template directed extension of nucleic acids or nucleic acid analogues by posing sterical hindrances to polymerases where extension is unwanted.

In another preferred embodiment oligonucleotide and/or oligonucleotide analogues comprising intercalator pseudonucleotides according to the present invention reduce the formation of unwanted extension products arising from cross-hybridization between oligonucleotides and oligonucleotide analogues in template directed extension of nucleic acids or nucleic acid analogues.

Above, said unwanted extension products refer to products arising from extension of said oligonucleotide and/or oligonucleotide analogues where non-target nucleic acids or nucleic acid analogues (including oligonucleotide and/or oligonucleotide analogues) serve as templates.

Furthermore, reduction of the formation of unwanted extension products should be interpreted as the situation compared to extension where all conditions for extension are identical except that said oligonucleotide and/or oligonucleotide analogues do not comprise intercalator pseudonucleotides according to the present invention.

Another strategy to reduce the formation of unwanted extension products relates to the cases where oligonucleotide and/or oligonucleotide analogues according to the present invention comprise a first sequence comprising intercalator pseudonucleotides, a second and a third sequence, wherein said first sequence is capable of hybridizing to said second sequence. When the first and second sequences are homologously complementary, they will preferably form a hairpin structure with a duplex without overhang where the 5' and 3' ends of the oligonucleotide and/or oligonucleotide analogue meet. The 3' end of such a structure cannot be extended, and if said structure is stable it efficiently prevents unwanted cross-hybridization to other nucleic acids or nucleic acid analogues and therefore the formation of unwanted extension products. Preferably, the melting temperature of a hybrid of said second and third sequence and a homologously complementary target sequence is above the melting temperature of a hybrid between the first sequence and the second sequence. Thus, when target is present, the oligonucleotide and/or oligonucleotide analogue will preferably hybridize with said target and prime an extension reaction. If said target is not present, said first and second sequence will hybridize forming a hairpin thus protecting from cross-hybridization and the formation of unspecific products.

In one embodiment an oligonucleotide and/or oligonucleotide analogue comprising intercalator pseudonucleotides according to the present invention the duplex formed between the first and second sequence is without overhang.

- 5 In another embodiment an oligonucleotide or oligonucleotide analogue comprising intercalator pseudonucleotides according to the present invention the duplex formed between the first and second sequence has a 3'-overhang.

10 It is preferred that homologously complementary first and second sequences form duplexes, preventing unwanted extension events when the oligonucleotide and/or oligonucleotide analogue is not hybridized to a target sequence in a template or when oligonucleotide and/or oligonucleotide analogue sequences is not present or present in limiting amounts. Intramolecular duplex formation will happen fast due to the superior kinetics of the uni-molecular hybridization event thus obtained during  
15 intramolecular duplex-formation when said template is not present. When fluorescence properties of oligonucleotide and/or oligonucleotide analogues are used for detection of extension reactions, intercalator pseudonucleotides capable of forming intramolecular excimers, intramolecular exciplexes, FRET complexes or charge-transfer complexes can be positioned in the first sequence of said  
20 oligonucleotide and/or oligonucleotide analogue. By choosing this design, the fluorescence signal from said intercalator pseudonucleotides is quenched upon the formation of a duplex comprising said first and second sequence. In this way, the fast kinetics of uni-molecular hairpin formation provides means of switching between fluorescence on-off states fast. For example fast kinetics are preferred to minimize  
25 PCR cycling time.

Typically, increasing the number of different nucleic acids and/ or nucleic acid analogues in especially PCR reactions leads to the formation of a large increase in the number of unwanted extension products. This is due to the increased number of  
30 possible combinations of unspecific hybridization events between said nucleic acids and/ or nucleic acid analogues. Therefore, the methods of effectively reducing or removing unwanted extension products as described above also allows for more optimal PCR multiplexing.

Excimers, exciplexes, FRET complexes and charge transfer complexes often have the property that the excitation- and emission-peaks are widely separated (measured in nm). The nm separation between excitation- and emission-peaks are referred to as "the Stokes shift" and typical examples of Stokes shifts found for  
5 conventional fluorophores used for quantitative PCR are in the range of 10-25 nm. For the pyrene excimer, the Stokes shift is approximately 140 nm. It is preferred to have large Stokes shifts to facilitate separation of excitation light from detected light to avoid false signal and to avoid cross-talk between fluorophores where one fluorophore excites another independently from amplified nucleic acid or nucleic acid  
10 analogue.

Accordingly, it is an preferred object of the present invention to provide oligonucleotides or oligonucleotide analogues comprising intercalator pseudonucleotides according to the present invention capable of forming  
15 intramolecular excimer, and/or intramolecular exciplex, and/or FRET and/or charge transfer complex fluorescence with Stokes shifts significantly larger than the non-intercalating pseudonucleotide fluorophores mentioned herein above.

In one embodiment of the present invention an oligonucleotide or oligonucleotide  
20 analogue comprises modifications in backbone moieties not being derived from nucleotides, nucleotide analogues and/or intercalating pseudonucleotides according to the present invention. Such modifications are capable of being build into an oligonucleotide or an oligonucleotide analogue backbone but are not capable of taking part in base-pairing events or in any autonomous way contribute to increased  
25 melting hybridization strength of a nucleic acid and/or nucleic acid analogue

Preferebly said backbone modifications are small chemical moieties capable of adjoining two nucleobases in a nucleic acid or nucleic acid analogue sequence without essentially disturbing the base-pairing properties of said sequence nucleic  
30 acid or nucleic acid analogue. By inserting said backbone modifications into the backbone of a nucleic acid or nucleic acid analogue, small backbone bulges are thus obtained between nucleobases. This provides the backbone of the nucleic acid or nucleic acid analogue with increased local conformational flexibility.



Examples of backbone modifications are described herein above, since said backbone modifications largely equals the backbone moieties of intercalating pseudonucleotides.

- 5 Said flexible backbone units can be inserted at any internal position within a nucleic acid or nucleic acid analogue or a pair of nucleic acids and/or nucleic acid analogues in any distance from intercalating pseudonucleotides if present. Preferably, said flexible backbone modifications are placed such that they are positioned opposite of intercalator pseudonucleotides when both intercalator  
10 pseudonucleotides and said backbone modifications are present within a duplex or hybrid structure.

- More preferably said flexible backbone moieties, when placed directly opposite of an intercalator pseudonucleotide in a duplex structure, are capable of accommodating  
15 said intercalator pseudonucleotide with better fit than when said backbone moiety is not present. In this regard, a better fit means that the increased flexibility of the opposite backbone in a duplex will allow the intercalator to adopt a stable conformation with a more favorable energy minimum than when the opposite strand does not comprise said backbone moiety.

- 20 In another preferred embodiment at least two intercalating pseudonucleotides capable of forming an excimer, exciplex, FRET complex or charge-transfer complex are positioned as bulge insertions in a oligonucleotide or oligonucleotide analogue sequence. When said sequence forms a hybrid with a complementary nucleic acid  
25 analogue, each of said intercalator pseudonucleotides are positioned opposite of a flexible backbone moiety. In a more preferred embodiment, most or all of said intercalator pseudonucleotides are accommodated with better fit, and in the most preferred embodiment, the increased flexibility in accommodation results in a significant decrease in excimer, exciplex, FRET or charge-transfer fluorescence of  
30 intercalator pseudonucleotides present in the duplex structure.

- Accordingly, an increased efficiency whereby excimer, exciplex, FRET or charge-transfer fluorescence is quenched when present in a duplex structure provides for an increased signal-to-noise ratio of fluorescence detection systems according to  
35 the present invention.

Said complementary nucleic acid analogue sequence comprising flexible backbone moieties need not be a separate sequence, but can be covalently linked to said first sequence, according to the hairpin oligonucleotide and oligonucleotide analogue designs described above. In such embodiments said hairpin may both function as an hybridization probe and/or a primer for an elongation process, such as PCR.

#### Method of detecting hybridisation

- 10 The present invention also relates to methods of detecting hybridisation between a nucleic acid or nucleic acid analogue comprising an oligonucleotide or oligonucleotide analogue and a template comprising a nucleic acid sequence capable of hybridising to the second sequence comprising the steps of
- 15 g) providing the template; and  
h) providing an oligonucleotide or oligonucleotide analogue as described herein above; and  
i) incubating the template and the oligonucleotide or oligonucleotide analogue under conditions allowing for hybridisation; and  
20 j) detecting hybridisation.

The nucleic acid or nucleic acid analogue comprising an oligonucleotide or oligonucleotide analogue may in one embodiment be equal to said oligonucleotide or oligonucleotide analogue. However, it is also possible, that the nucleic acid or nucleic acid analogue comprising an oligonucleotide or oligonucleotide analogue may be longer than the oligonucleotide or oligonucleotide analogue.

For example the nucleic acid or nucleic acid analogue may be an elongated oligonucleotide or oligonucleotide analogue, wherein nucleotides have been added to the 3' end of the oligonucleotide or oligonucleotide analogue. An elongated oligonucleotide or oligonucleotide analogue may be the product of a reaction catalysed by a DNA polymerase.

In particular, a DNA polymerase may elongate an oligonucleotide or oligonucleotide analogue, when said oligonucleotide or oligonucleotide analogue is hybridised to a longer homologously complementary nucleic acid in a template dependent manner. Preferably said homologously complementary nucleic acid may be DNA or RNA and  
5 more preferably said homologously complementary nucleic acid might be the template.

Accordingly, in one embodiment said nucleic acid or nucleic acid analogue may be a product of a PCR reaction comprising said oligonucleotide or oligonucleotide  
10 analogue.

Hybridisation may be determined by a number of different techniques taking advantage of the properties of the oligonucleotide or oligonucleotide analogue.

15 In one embodiment, detection of hybridisation comprises determining spectral properties of the oligonucleotide or oligonucleotide analogue. Determination of spectral properties is described in more detail herein above.

Alternatively, hybridisation may be determined by determining the melting  
20 temperature. This may be done when the melting temperature of a hybrid between the first sequence and the second sequence is different to the melting temperature of a hybrid consisting of the second sequence and a homologously complementary nucleic acid or nucleic acid analogue for example the template.

25 Further, said nucleic acid or nucleic acid analogue comprising said oligonucleotide or oligonucleotide analogue not hybridised to said template may be separated from said hybridised nucleic acid or nucleic acid analogue prior to detection by any method known to the person skilled in the art.

30 The method of detecting hybridisation may be used for a number of different purposes. For example, the method may be employed for detecting hybridisation in an assay dependent on specific hybridisation, for example Southern Blotting, Northern blotting, FISH or other kinds of in situ hybridisation.

However, in a preferred embodiment the method is employed for quantification of a polymerase chain reaction (PCR).

5 In yet another embodiment said nucleic acid analogue comprising said oligonucleotide or oligonucleotide analogue or said template may be affixed to a solid support.

Method of detecting template directed extension

10 The present invention also relates to methods of detecting template directed extension primed by oligonucleotide and/or oligonucleotide analogues.

- a) providing a mixture comprising the template; and
- b) providing an oligonucleotide and/or oligonucleotide analogue as described herein above; and
- 15 c) incubating the template and the oligonucleotide and/or oligonucleotide analogue under conditions allowing for hybridization; and
- d) incubating the template and the oligonucleotide and/or oligonucleotide analogue under conditions allowing for template directed extension; and
- e) optionally separating extended oligonucleotide and/or oligonucleotide  
20 analogues from essentially non-extended oligonucleotide and/or oligonucleotide analogues
- f) detecting extension.

25 For example the template may be an extended/elongated oligonucleotide or oligonucleotide analogue, wherein nucleotides have been added to the 3' end of an oligonucleotide or oligonucleotide analogue. An elongated oligonucleotide or oligonucleotide analogue may be the product of a reaction catalysed by a DNA polymerase or a ligase.

30 In particular, a DNA polymerase may elongate an oligonucleotide analogue, when said oligonucleotide analogue is hybridized to a longer nucleic acid or nucleic acid analogue in a template dependent manner. Preferably said nucleic acid or nucleic acid analogue may be DNA or RNA and more preferably said nucleic acid might be the template.

In one embodiment, detection of extension comprises determining spectral properties of the oligonucleotide analogue. One advantage of such a detection type is that it can be done real-time during a reaction, so that the amount of extended  
5 oligonucleotide analogue can be determined at any specific time during a reaction. The reaction may for example be a PCR.

Dependent on the kind of spectral properties that are determined, a spectral signal above or below a predetermined limit may be indicative of extension, preferably  
10 however, a spectral signal above a predetermined limit is indicative of extension.

The spectral properties may for example be fluorescent properties. In a preferred embodiment the spectral properties are monomer fluorescence and/or excimer fluorescence and/or exciplex fluorescence and/or FRET fluorescence and/or charge-  
15 transfer absorbance.

It is an embodiment of the present invention to provide oligonucleotide analogues comprising at least 2 intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or a FRET complex and/or charge-transfer  
20 complex as described herein above.

In an preferred embodiment said oligonucleotide analogues comprising a first sequence comprising at least 2 intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or FRET and/or charge-transfer complex  
25 as described herein above are employed.

In another preferred embodiment said oligonucleotide or oligonucleotide analogue is affixed to a solid support.

30 In another more preferred embodiment said oligonucleotide or oligonucleotide analogue is affixed to a solid support and capable of priming an extension reaction when said oligonucleotide or oligonucleotide is hybridised to a corresponding, preferentially a complementary, target nucleic acid or nucleic acid analogue.

35 Method for detecting extension when using linear oligonucleotide analogues

5 In one embodiment the oligonucleotide analogue according to the present invention preferably comprises at least one intercalator pseudonucleotide capable of fluorescing, more preferably the oligonucleotide analogue comprises only intercalator pseudonucleotide capable of fluorescing.

10 In one embodiment monomer fluorescence of intercalator pseudonucleotides is used for detection of extension of oligonucleotide analogues according to the present invention.

In another embodiment the monomer fluorescence of an intercalator pseudonucleotide positioned at the 5' end of the second sequence, where the second sequence is positioned downstream of the first sequence, is used to detect extension of oligonucleotide analogue.

15 More preferably oligonucleotide analogues comprise at least 2 intercalator pseudonucleotides capable of forming an intramolecular excimer and/or intramolecular exciplex and/or FRET and/or charge-transfer complex.

20 In one embodiment extension of the second downstream sequence may be determined by determining the excimer, exciplex, FRET or charge-transfer fluorescence. Here high excimer, exciplex, FRET or charge-transfer fluorescence is indicative of template directed extension and low excimer, exciplex, FRET or charge-transfer fluorescence is indicative of no template directed extension.

25 High fluorescence is fluorescence above a predetermined limit and low fluorescence is fluorescence below a predetermined limit.

30 In addition it is possible to detect hybridization by determining fluorescence of one or more fluorescent groups attached to the oligonucleotide analogue, preferentially in the first sequence.

Accordingly, when fluorescence of the fluorescent group is detectable over a predetermined limit, that is indicative of no extension directed from the template,

whereas when fluorescence of the fluorescent group is lower than a predetermined limit, which is indicative of no extension.

5 In one embodiment, said at least 2 intercalator pseudonucleotides of the first sequence are capable of co-stacking with the end of the hybrid comprised of an extended oligonucleotide analogue and a homologously complementary template.

10 In a preferred embodiment, said at least 2 intercalator pseudonucleotides of the first sequence are capable of co-stacking with the end of the hybrid comprised of an extended oligonucleotide analogue and a complementary template.

15 In one embodiment, non-extended oligonucleotide analogue is differentiated from extended oligonucleotide analogue by fluorescence polarization or fluorescence correlation spectroscopy.

In another embodiment non-extended oligonucleotide analogue is differentiated from extended oligonucleotide analogue by the use of one or more fluorescent groups that give rise to a signal specific to extended oligonucleotide analogues only.

20 For example fluorescent dyes that are attached to the oligonucleotide and/or oligonucleotide analogues and are specific for double stranded nucleic acids or nucleic acid analogues may be used. When the temperature is sufficiently high allowing only homologously complementary hybrids of oligonucleotide analogue comprising nucleic acid analogues to hybridize, such signal may be used for  
25 detection. Signal derived from long unspecific extension products may give problems, but that may be removed by using methods for reducing unspecific hybridization and extension events as described above.

30 Fluorescent dyes specific for double stranded nucleic acid or nucleic acid analogue that are not covalently attached to said oligonucleotide or oligonucleotide analogue may also be used. As described herein above, such dyes will simply stain all double stranded nucleic acid or nucleic acid analogue formed during the amplification reaction being present on double stranded form at the chosen measuring  
35 temperature.

In one embodiment concerning the oligonucleotide or oligonucleotide analogue comprising a first and a second sequence, non-extended oligonucleotide or oligonucleotide analogue is separated from extended oligonucleotide or oligonucleotide analogue prior to detection.

5

Separation of non-extended from extended oligonucleotide or oligonucleotide analogues is only carried out when the signal from labels associated with non-extended and extended oligonucleotide or oligonucleotide analogue cannot otherwise be differentiated. Separation may for example be carried out by gel

10

electrophoresis, chromatography, centrifugation, precipitation or any other method known to the person skilled in the art.

Method for detecting extension and/or hybridisation when using hairpin oligonucleotide analogues

15

The present section concerns oligonucleotide and/or oligonucleotide analogues comprising a first, a second and possibly a third sequence, wherein said first sequence is capable of hybridizing to said second sequence, and wherein the first sequence may comprise at least 2 intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or FRET and/or charge-transfer complex as described herein above.

20

Accordingly, in one embodiment hybridisation of the second sequence and/or In addition it is possible to detect hybridization by determining fluorescence of non-intercalator pseudonucleotides attached to the oligonucleotide and/or oligonucleotide analogue. In particular, it is possible when oligonucleotide and/or oligonucleotide analogues comprise a fluorescent group and a quencher molecule as described herein above.

25

For example the oligonucleotide and/or oligonucleotide analogue may comprise a fluorescent group and a quencher molecule positioned in relation to each other so that the quencher molecule is capable of quenching the fluorescence of the fluorescent group when the first sequence is hybridized to the second sequence, but is not capable of quenching the fluorescence of the fluorescent group when the first sequence is not hybridized to the second sequence.

30

35



Accordingly, when fluorescence of the fluorescent group is detectable over a predetermined limit, that is indicative of no hybridization (i.e. no hybridization between first and second sequence) due to extension directed from the template, whereas when fluorescence of the fluorescent group is lower than a predetermined limit, that is indicative of no extension allowing hybridization (i.e. hybridization between first and second sequence).

High fluorescence is fluorescence above a predetermined limit and low fluorescence is fluorescence below a predetermined limit.

In a preferred embodiment said oligonucleotide or oligonucleotide analogue is affixed to a solid support.

Method of detection hybridisation and/or extension when using melting temperature

Alternatively, the presence of extension product and/or hybridisation may be determined by determining the melting temperature. This may be done when the melting temperature of a hybrid between the first sequence and the second sequence of an oligonucleotide analogue is lower than the melting temperature of an extension product of said oligonucleotide analogue and a homologously complementary or complementary nucleic acid or nucleic acid analogue, for example the template.

Accordingly, low melting temperature is indicative of intramolecular hybridization between first and second sequence and essentially no extension, whereas high melting temperature is indicative of the presence of extension products.

Hence a melting temperature above a predetermined limit may be indicative of extension.

In a preferred embodiment of the present invention the oligonucleotide analogue is designed in a manner so that only part of the oligonucleotide analogue is capable of hybridizing with the preferred template.

For example, in one embodiment the template does not comprise a nucleic acid sequence capable of hybridizing with the first sequence. In another embodiment the template does not comprise a nucleic acid sequence capable of hybridizing with for example 1 to 15, such as 1 to 10, for example 1 to 7, such as 1 to 5, for example 1 to 3 nucleotides flanking the intercalator pseudonucleotide(s). In yet another embodiment the template does not comprise nucleic acid sequences that may hybridize with the part of the first sequence, which is capable of hybridizing to the second sequence.

10 Preferably the template comprises a nucleic acid sequence capable of hybridizing with the second sequence and the third sequence, more preferably the template may comprise a nucleic acid sequence complementary to the second sequence and the third sequence.

15 Furthermore, the template may comprise a nucleic acid sequence capable of hybridizing with the part of the first sequence that is not capable of hybridizing to the second sequence.

20 Most preferably the template does not comprise a nucleic acid sequence capable of hybridizing with the first sequence and the template comprises a nucleic acid sequence capable of hybridizing with the second sequence and the third sequence.

25 In said embodiment, when the oligonucleotide or oligonucleotide analogue is hybridized to the template, the first sequence is not hybridized. Accordingly, when the first sequence comprises intercalators capable of forming an intramolecular excimer and/or intramolecular exciplex and/or intramolecular FRET and/or intramolecular charge-transfer complex, then excimer fluorescence and/or exciplex fluorescence and/or FRET and/or charge-transfer absorbance may be detected, when the oligonucleotide or oligonucleotide analogue is hybridized to the template.

30 Because it is preferred that the second sequence preferentially hybridizes with a homologically complementary template, the first and the second sequence will only be hybridized, when no homologically complementary template is present. Accordingly, when a homologically complementary template is available the second sequence will preferably be hybridized with said homologically complementary

35

template and hence the first sequence will not be hybridized and excimer fluorescence and/or exciplex fluorescence and/or FRET and/or charge-transfer complex fluorescence above a predetermined limit may be detected.

- 5      However, in a preferred embodiment the method is employed for quantification of a polymerase chain reaction (PCR). Because the method allows for real-time detection of hybridization by determining fluorescence properties, the method allows to closely follow a PCR reaction as it is proceeding.

## Examples

The following examples illustrate selected embodiments of the invention and should not be regarded as limiting for the invention.

5

In the examples the following abbreviations are used:

ODN: Oligodeoxynucleotide

10

INA: Intercalating nucleic acid corresponding to intercalator pseudonucleotide

### Example 1

#### **Preparation of an intercalator pseudonucleotide**

15

1-Pyrenemethanol is commercially available, but it is also easily prepared from pyrene by Vilsmeier-Haack formylation followed by reduction with sodium borohydride and subsequent conversion of the alcohol with thionyl chloride affords 1-(chloromethyl)pyrene in 98% yield.

20

The acyclic amidite **5** (fig. 1) was prepared from (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol and 1-(chloromethyl)pyrene in 52% overall yield. The synthesis of **5** (fig. 1) is accomplished using KOH for the alkylation reaction, and using 80% aqueous acetic acid to give the diol **3** (fig. 1), which is protected with dimethoxytritylchloride (DMT-Cl) and finally reaction with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite affords target compound **5** (fig. 1) in 72% yield. The yield in the latter reaction step was decreased from 72% to 14% when 2-cyanoethyl *N,N*-diisopropylchlorophosphor amidite was used as the phosphitylating reagent. The synthesis of the acyclic amidite **5** is shown schematically in figure 1.

25

30

#### 1-Pyrenylcarbaldehyde

A mixture of *N*-formyl-*N*-methylaniline (68.0 g; 41.4 mL; 503 mmol) and *o*-dichlorobenzene (75 mL) is cooled on an ice bath and added phosphoroxychloride

(68g; 440 mmol) over 2 hours so that the temperature do not exceed 25 °C. Pulverized Pyrene (50 g; 247 mmol) is added in small portions over 30 min. and the reaction mixture is equipped with a condenser and heated at 90-95°C for 2 hours. After cooling to room temperature the dark red compound is filtered off and washed with benzene (50 mL.). Then it is transferred to water (250 mL) and stirred over night. The yellow aldehyde is filtered of and washed with water (3×50 mL). Recrystallized from 75% ethanol 3 times. Yeild: 30.0 g (52.7%).

10 1-Pyrenylmethanol

1-Pyrenylcarbaldehyde (10.0 g; 43.4 mmol) is dissolved in dry THF (50 mL) under inert atmosphere and NaBH<sub>4</sub> (0.82 g; 22 mmol) is added in small portions over 10 min. The reaction mixture is stirred over night at r.t. and crystallizing the product by pouring into stirring water (350 mL). The product is filtered off, washed with water (4×25 mL) and dried under reduced pressure. Recrystallized from ethyl acetate. Yeild: 8.54 g (84.7%).

1-(Chloromethyl)-pyrene

1-Pyrenylmethanol (6.40 g; 27.6 mmol) is dissolved in a mixture of pyridine (3.3 mL; 41.3 mmol) and CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and the mixture is cooled to 0°C. SOCl<sub>2</sub> (3.0 mL; 41.3 mmol) is added slowly over 15 min. and the temperature is allowed to rise slowly to r.t. Stir over night. The mixture is poured into stirring water (200 mL) and added CH<sub>2</sub>Cl<sub>2</sub> (100 mL). The mixture is stirred for 30 min. The organic phase is washed with 5% NaH<sub>2</sub>CO<sub>3</sub> (2×75 mL) and brine (2×75 mL) respectively, dried with sodium sulfate and concentrated under reduced pressure. Recrystallized from toluene/ petroleum ether. Yield 6.75 g (97.8%).

(S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol

Pulverized KOH (25 g) and 1-(Chloromethyl)-pyrene (6.0 g; 23.9 mmol) is added to a solution of (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (2.6 g; 19.7 mmol) in dry toluene (250 mL). The mixture is refluxed under Dean-Stark conditions in 16h, then cooled to r.t. and added water (150 mL). The organic phase is washed with water (3×100 mL) dried with a combination of magnesium sulfate and sodium sulfate and concentrated under reduced pressure to a thick oil. Silica gel chromatography (CH<sub>2</sub>Cl<sub>2</sub>) afforded the pure compound in 6.1 g (90%).

(R)-3-(1-Pyrenemethoxy)-propane-1,2-diol

(S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (6.1 g; 17.6 mmol) is added to a mixture of acetic acid and water (100 mL; 4:1) and is stirred at r.t. for 19h. Concentrated under reduced pressure. Giving an oil in quantitatively yield.

(S)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol

(R)-3-(1-Pyrenemethoxy)-propane-1,2-diol (760 mg; 2.48 mmol) is dissolved in dry pyridine (20 mL) and added dimethoxytrityl chloride (920 mg; 2.72 mmol). The reaction mixture is stirred in 24h and concentrated under reduced pressure. Purified by silica gel chromatography (ethyl acetate/ cyclohexane/ triethylamine 49:49:2) to give a white foam. Yield 1.20 g (79.5%).

Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol

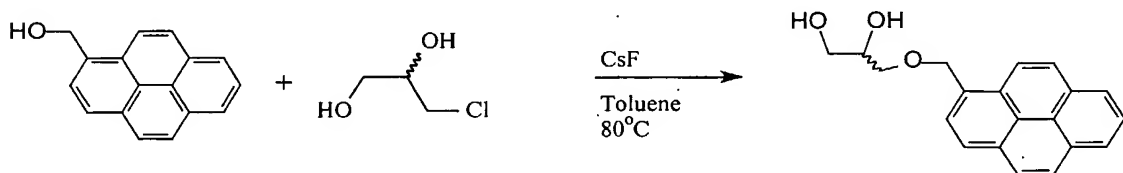
(S)-1-(4,4'-Dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol (458 mg; 753 μmol), 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphan (453 mg; 429 μL; 1.51 mmol) and diisopropylammonium tetrazolid (193 mg; 1.13 mmol) is mixed in dry CH<sub>2</sub>Cl<sub>2</sub> (7 ml) and stirred under nitrogen atmosphere for 6 days. Purified by silica gel

chromatography (ethyl acetate/ cyclohexane/ triethylamine 49:49:2) and dried under reduced pressure. Yield 438 mg (72%) as a white foam.

### Example 2

5

#### Alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol



**Scheme 1:** Alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol

10

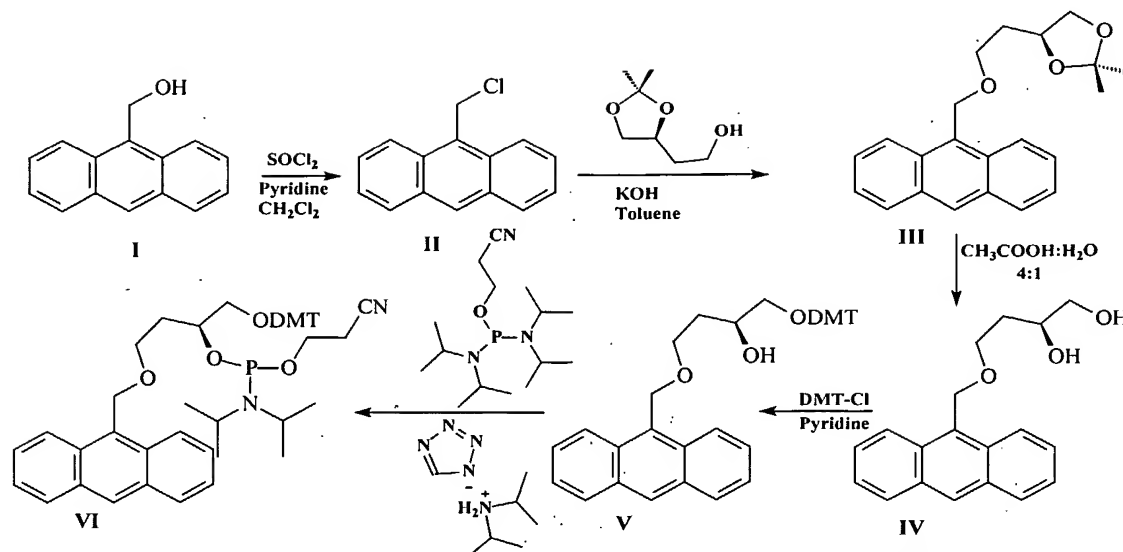
1-Pyrenylmethanol (232 mg; 1.0 mmol) is dissolved in hot toluene (2 mL over Na). CsF (7 mg; 0,046 mmol) is added and stirred for approx. 1h at room temperature when 3-chloro-1,2-propanediol (170 mg; 1.53 mmol) is added. The mixture is stirred at 80°C for 2h, cooled off to room temperature and the precipitated product is separated from the mixture by filtration. Washed with cold toluene (2 × 1 mL). Yield 220 mg (72%).

15

### Example 3

20

**Synthesis of the 2-O phosphoramidite of 1-O-4,4'-dimetoxytrityl-4-O-(9-antracenylmethyl)-1,2,4-butanetriol**



**Scheme 2: Schematic view of the synthesis of the 2-O-phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-antracenylnmethyl)-1,2,4-butanetriol**

5 9-anthracenemethylchlorid (II)

9-anthracenemethanol (0.81 g; 3.89 mmol; I) was dissolved in dry pyridine (467  $\mu$ L; 5.83 mmol) and dry  $\text{CH}_2\text{Cl}_2$ . Under stirring and at  $0^\circ\text{C}$   $\text{SOCl}_2$  (423  $\mu$ L; 5.83 mmol) was added dropwise, and the mixture was stirred for 24h during which the temperature is allowed to rise to r.t. within 2h. The reaction is poured onto stirring  $\text{H}_2\text{O}$  (60 mL) and was added additional  $\text{CH}_2\text{Cl}_2$  (40 mL). The organic phase was washed with a 5%  $\text{NaHCO}_3$  (100 mL) solution, brine (100 mL) and water (100 mL) respectively. Dried over  $\text{Na}_2\text{SO}_4$  and concentrated *in vacuo*. Yield 665 mg (75%).

15 1,2-D-isopropylidene-4-(9-anthracenylmethyl)-1,2,4-butanetriol (III)

9-anthracenemethylchlorid (628 mg; 2.77 mmol) was dissolved in dry toluene (25 mL over Na) and 2-[(S)-2',2'-dimethyl-1',3'-dioxolan-4'-yl]-ethanol (506 mg; 3.5 mmol) and 3 small spoons of KOH was added. The mixture was connected to a Dean-Stark apparatus and stirred under reflux conditions over night. The reaction mixture was slowly cooled to r.t. and washed with  $\text{H}_2\text{O}$  (4  $\times$  25 mL). Dried over  $\text{Na}_2\text{SO}_4$  and concentrated *in vacuo*.

4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (IV)



To the dried compound was added 80% AcOH (50 mL) and the reaction mixture was stirred in 24h at r.t. The mixture was concentrated in vacuo over night and purified by short, fast silica gel chromatography (impurities was first eluated with CH<sub>2</sub>Cl<sub>2</sub>, and product was then eluated with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Yield 56.3% over 2 steps.

1-O-(4,4'-dimethoxytrityl)-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (V)

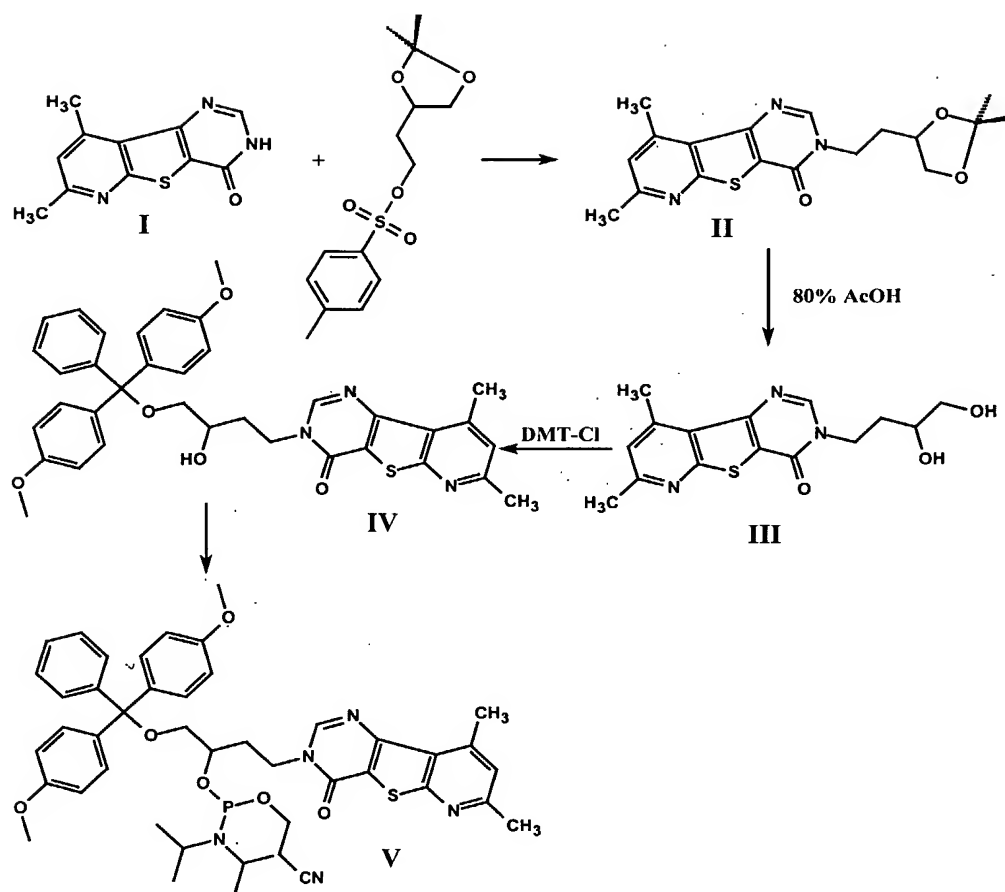
The diol (425 mg; 1.40 mmol) and DMT-Cl is mixed in dry pyridine (5 mL) and stirred at r.t. for 36h. The reaction mixture was concentrated in vacuo and purified by silica gel chromatography (EtOAc:C<sub>6</sub>H<sub>12</sub>:N(Et)<sub>3</sub> 63:35:2). Co-evaporated with ether (5 mL over Na) after which a yellowish foam was isolated. Yield 630 mg (74%).

The phosphoramidite of 1-O-(4,4'-dimethoxytrityl)-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (VI)

The DMT protected anthracene compound was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (7 mL) and diisopropylammonium tetrazolide (252 mg; 1.5 mmol) and 2-Cyanoethyl N,N,N',N'-tetraisopropyl Phosphane was added. The reaction mixture was stirred for 20h at r.t. Concentrated in vacuo and purified by silica gel chromatography (EtOAc:C<sub>6</sub>H<sub>12</sub>:N(Et)<sub>3</sub> 24:74:2). Co-evaporated with ether (5 mL over Na) to give a yellowish foam (67%).

Example 4

**Synthesis of the Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)**



**Scheme 3: Synthesis of the Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)**

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (I)

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one was prepared according to literature procedures.<sup>1,2,3</sup>

3-N-((S)-2'',2''-dimethyl-1'',3''-dioxalane-4''-ethanyl)-7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (II)

<sup>1</sup> Schimdt, U. & Kubitzek, H., Chem. Ber., 93, 1559 (1960)

<sup>2</sup> Hassan, K. M. et al. Phosphorous, Sulfur, Silicon Relat. Elem., 47, 181 (1990)

<sup>3</sup> Gewald, K. & Jansch, H. J., Prakt. Chemie 313-320 (1976)

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (1.16 g; 5.0 mmol) was suspended in anhydrous DMF (20 mL) and NaH (0.2 g; 5.0 mmol, 60% dispersion in mineral oil) was added. The mixture was stirred for 2h until all H<sub>2</sub> evolving ceased. Then (S)-2,2-dimethyl-1,3-dioxalane-4-ethanoyl-O-para-toluenesulfonate (0.78 g; 5.1 mmol) was added in one portion and the mixture was stirred for 24h at 80°C. The mixture was evaporated to dryness *in vacuo*, co evaporated with dry toluene (3 × 10 mL) *in vacuo* and the residue was purified silica gel chromatography (5% EtOAc in CHCl<sub>3</sub>) to get a colorless product. Yield 0.81 g; 45%.

10 (S)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-butan-1,2-diol (III)

3-N-((S)-2'',2''-dimethyl-1'',3''-dioxalane-4''-ethanyl)-7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (0.75 g; 2.1 mmol) was stirred at r.t. in 80% AcOH (20 mL) for 24h. The product was obtained by concentration *in vacuo* and co-evaporation with EtOH. Purified by silica gel chromatography (5% MeOH in CHCl<sub>3</sub>) to get the colorless product. Yield 0.5 g (75%).

20 (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (IV)

(S)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-butan-1,2-diol (0.6 g; 1.9 mmol) was dissolved in dry pyridine (5 mL) and DMT-Cl (0.71 g; 2.1 mmol) was added. Stirred at r.t. over night. Concentrated *in vacuo* and co evaporated using dry toluene (3 × 10 mL). The residue was purified by silica gel chromatography (EtOAc:C<sub>6</sub>H<sub>12</sub>:N(Et)<sub>3</sub> 49:49:2) to yield a white foam. Yield 0.77 g (65%)

30 Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)

(S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (310 mg; 0.5 mmol) was dissolved under nitrogen in anhydrous dichloromethane (10 mL). Diisopropylammoniumtetrazolide (0.11 g; 0.67 mmol) was added followed by dropwise addition of 2-Cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.3 g; 1.0 mmol) the reaction was stirred over night

under nitrogen atmosphere, concentrated *in vacuo* and purified by silica gel chromatography (EtOAc:<sup>c</sup>C<sub>6</sub>H<sub>12</sub>:N(Et)<sub>3</sub> 49:45:12) to give a white foam. Yield 345 mg (84%)

## 5 Example 5

### **Duplexes with dangling ends.**

10 To investigate the stacking ability of the nucleoside analogue 5 (fig. 1) it was incorporated into the 5' end of two different self-complementary strands (5'-XCGCGCG and 5'-XTCGCGCGA).

15 The ODN synthesis is carried out on a Pharmacia LKB Gene Assembler Special using Gene Assembler Special software version 1.53. The pyrene amidite is dissolved in dry acetonitrile, making a 0.1M solution and inserted in the growing oligonucleotides chain using same conditions as for normal nucleotide couplings (2 min. coupling). The coupling efficiency of the modified nucleotides is greater than 99%. The ODNs are synthesized with DMT on and purified on a Waters Delta Prep 3000 HPLC with a Waters 600E controller and a Waters 484 detector on a Hamilton PRP-20 1 column. Buffer A: 950 ml. 0.1 M NH<sub>4</sub><sup>+</sup>HCO<sub>3</sub><sup>-</sup> + 50 ml MeCN pH =9.0; buffer B: 250 ml. 0.1 M NH<sub>4</sub><sup>+</sup>HCO<sub>3</sub><sup>-</sup> + 750 ml MeCN pH = 9.0. Gradients: 5 min. 100% A, linear gradient to 100% B in 40 min., 5 min. with 100% B, linear gradient to 100% A in 1 min. and the 100% A in 29 min (product peak ≈ 37 min.). The ODNs were DMT deprotected in 925 μl H<sub>2</sub>O + 75 μl CH<sub>3</sub>COOH and purified by HPLC again using the 25 same column, buffer system and gradients (product peak ≈ 26 min.). To get rid of the liable salts, the ODNs were re-dissolved in 1 ml of water and concentrated *in vacuo* three times.

30 All oligonucleotides were confirmed by MALDI-TOF analysis made on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. The transition state analyses were carried out on a Perkin Elmer UV/VIS spectrometer Lambda 2 with a PTP-6 temperature programmer using PETEMP rev. 5.1 software and PECSS software package ver. 4.3. Melting temperature measurements of the self-complementary sequences are made in 1 M NaCl, 10 mM Na•Phosphate pH 7.0, 35 1.5 μM of each DNA strand. All other ODNs are measured in a 150 mM NaCl, 10

mM, Na•Phosphate, 1 mM EDTA pH 7.0, 1.5  $\mu$ M of each strand. All melting temperatures giving are with an uncertainty on  $\pm 0.5$  °C.

5 The Amber forcefield calculations were done in MacroModel 6.0 and 7.0 with water as solvent and minimization is done by Conjugant Gradient method. The starting oligonucleotide sequences for calculation with the inserted pyrenes is taken from Brookhavens Protein Databank, and modified in MacroModel before minimization is started. Lam and Au-Yeung solved a structure of a self-complementary sequence, equal to the one used in this work, by NMR. Their structure is prolonged with the  
10 pyrene amidite at the 5'-end of each strand and used for the structural calculations. The other sequence is a 13-mer highly conserved HIV-1 long terminal repeat region. G-7 is replaced by the pyrene amidite and calculations are made with and without an across lying C-nucleotide. The pyrene is placed in the interior of the duplex from the beginning. All bonds are free to move and to rotate.

15 The melting temperature of modified and unmodified, self-complementary DNA are shown in Figure 33. Incorporation of the pyrene amidite in the 5' end as a dangling end stabilises the DNA duplex with 19.2 °C – 21.8 °C (8.6 °C – 10.9 °C per modification) depending on the underlying base pair. The stabilizations of the duplexes  
20 due to incorporation of 5 at the 5' termini of the nucleic acid strands are similar to those found by Guckian et al. who inserted a pyrene nucleoside at the 5' termini of self complementary ODNs (oligo deoxynucleic acids). The stabilisation can be explained by calculations using "MacroModel" which predict a structure where the pyrene moiety interacts with both nucleosides in the underlying basepair (figure 2).

25

**Table 1. Melting temperatures of self-complementary sequences with 5' modification..**

		$T_m (^{\circ}\text{C})$	$\Delta T (^{\circ}\text{C})$
5'	C-G-C-G-C-G	41.0	
3'	G-C-G-C-G-C		
5'	T-C-G-C-G-C-G-A	46.9	
3'	A-G-C-G-C-G-C-T		
5'	X-C-G-C-G-C-G	62.8	21.8
3'	G-C-G-C-G-C-X		
5'	X-T-C-G-C-G-C-G-A	64.1	17.2
3'	A-G-C-G-C-G-C-T-X		

## 5 Example 6

**End-positioned intercalating pseudonucleotides - stabilisation dependent on intercalator-linker length**

## 10 **Introduction**

In this example is shown the dependence on linker length for the increase of affinity by the addition of intercalating pseudonucleotide to the 5'-end of an oligonucleotide. There is further more shown two examples of intercalating pseudonucleotides of

15 comparable stabilisation effect.

## **Material and Methods**

Oligonucleotides:

20

Probe I: 3'-CGA ACT CX

Probe II: 3'-CGA ACT CD

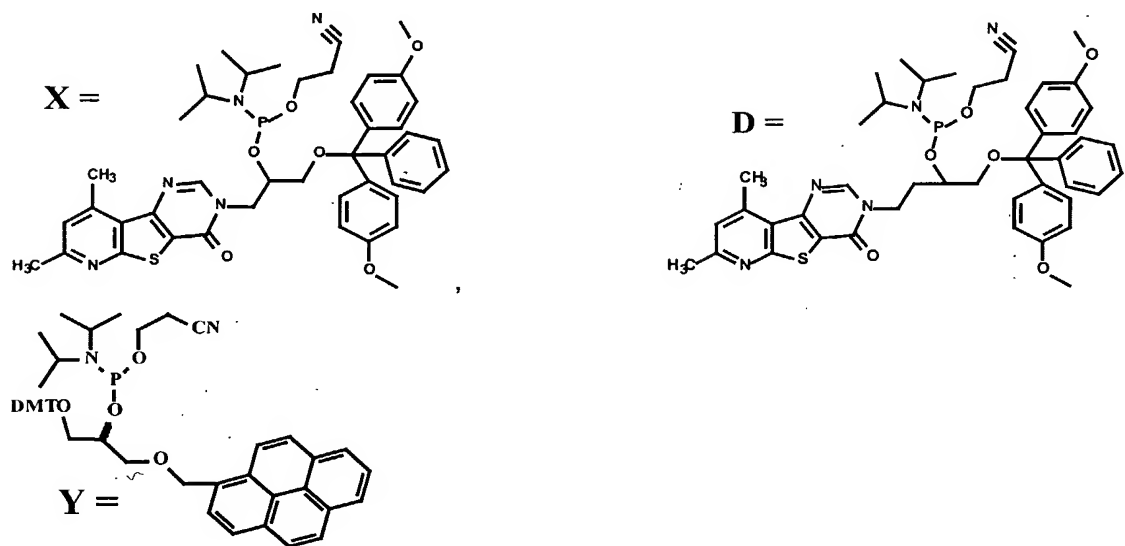
Probe III: 3'-CGA ACT CY

Ref: 3'-CGA ACT C

Target: 5'-GCT TGAG

Below is shown the amidites that were used in the preparation of the above mentioned oligonucleotides:

5



10

All hybridisation experiments were carried out with 1.5  $\mu\text{M}$  of both target and probe strands in 2 mL of a buffer solution containing:

140mM NaCl

10 mM  $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$

1 mM EDTA

15

pH = 7.0

20

The target strands and probes were annealed by mixing them in the above mentioned buffer at 95°C for 3 min. after which they are slowly cooled to room temperature. The melting temperatures of the hybridised probe-target hybrids were found by slowly heating the solution in a quartz cuvette, while simultaneously determining the absorbance. All melting temperatures presented in this example are with an uncertainty of  $\pm 1.0^\circ\text{C}$  as determined by repetitive experiments.

## Results and Discussion

The results of the melting experiments is shown in Table 5:

Name	Melting temperature (°C)	$\Delta T_m$ (°C)
Hybridisation to Target		
Ref	22.8	----
Probe I	28.4	5.6
Probe II	34.4	11.6
Probe III	33.8	11.0

The difference in melting temperature between probe I and II is due to the short linker of probe I. Hence it is important that the combined length of linker and intercalator is optimal, to obtain a large increase in affinity between intercalating pseudonucleotide modified oligonucleotides and their target DNA sequences. Probes II and III have nearly the same affinity for their target sequences, even though the intercalating moieties in the two probes are very different. This shows that the intercalating pseudonucleotides are a class of compounds that, dependent on the wanted feature it should introduce into an oligonucleotide or oligonucleotide analogue, it should be designed by more or less strict rules.

### Example 7

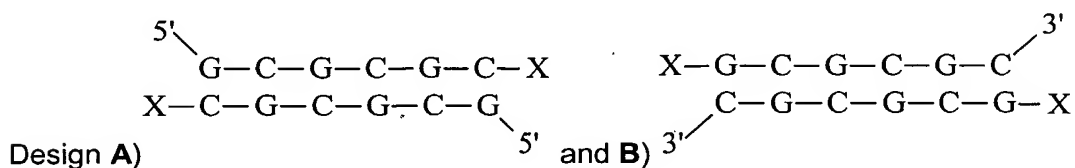
#### **Oligonucleotides synthesized on universal supports – obtaining 3' intercalator pseudonucleotide modified oligonucleotides**

In a preferred embodiment of the present invention, oligonucleotides or oligonucleotide analogues comprise intercalating pseudonucleotides at either or both ends. In this example it is shown that oligonucleotides or oligonucleotide analogues with intercalating pseudonucleotides in the 3'-end can be synthesised using Universal supports. It is furthermore shown that selfcomplementary oligonucleotides comprising intercalating pseudonucleotides positioned in the 3'-end form very thermal stable hybrids.

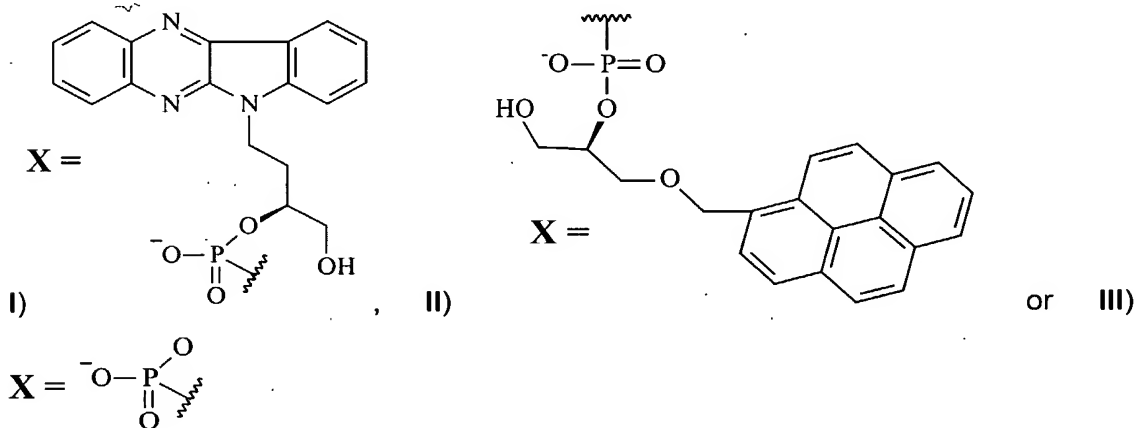


## Material and methods

The following two types of self-complementary probes were synthesised. Design A was synthesized using a universal support while B was synthesized using standard nucleotide coupled columns and procedures:



Two different intercalating pseudonucleotides were used for design A (I and II), and II was used for design B as well. One reference sequence without any intercalating pseudonucleotides (III) was synthesised. Hence X represents either:



After synthesis the oligo nucleotide analogues were treated with 2% LiCl in a 32% NH<sub>4</sub>OH solution in order to remove protection groups from the heterocyclic amines and to cleave the oligonucleotide from the universal support. Oligonucleotides comprising intercalating pseudonucleotides were tested on MALDI-TOF and found at the expected values.

All hybridisation experiments were carried out with 1.5 μM of both target and probe strands in 1 mL of a buffer solution containing:

140mM NaCl

10 mM Na<sub>2</sub>HPO<sub>4</sub>•2 H<sub>2</sub>O

1 mM EDTA

The target strands and probes were annealed by mixing them in the above mentioned buffer at 95°C for 3 min. after which they are slowly cooled to room temperature. The melting temperatures of the hybridised probe-target hybrids were found by slowly heating the solution in a quartz cuvette, while simultaneously determining the absorbance. All melting temperatures presented in this example are with an uncertainty of ± 1.0°C as determined by repetitive experiments.

## Results and discussion

<u>X\ Design</u>	A	B
I	57.3°C	-----
II	59.2°C	62.8°C
III	41.0°C	41.0°C

From the above table it can be seen that the insertion of intercalating pseudonucleotides in either end of an oligonucleotide increases the affinity for a complementary target nucleic acid. It is also shown that intercalating pseudonucleotides can be inserted into the 3' end of an oligonucleotide or oligonucleotide analogue by using standard universal base chemistry.

### Example 8

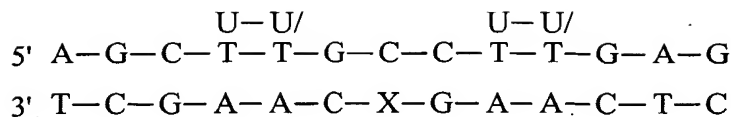
#### **Substituting a nucleotide with a 1-O-(pyrenylmethyl)glycerol nucleotide.**

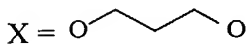
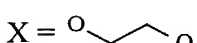
The ODN synthesis is carried out as described in example 5. Phosphoramidite 5 (fig. 1) is prepared as described in example 1.

UV melting temperature measurements (Table 2) where a G nucleotide is replaced by the flexible, abasic linkers ethylene glycol and 1,3-propandiol shows, not surpris-

ingly, a decrease in duplex stability compared to the unmodified, fully complementary sequence. The required DMT protected cyanoethyl N,N-diisopropylphosphoramidites of ethylene glycol and 1,3-propanediol were synthesized by standard methods. Having the 1-O-(pyrenylmethyl)glycerol nucleotide in the same position instead  
5 of the abasic diols increases the melting temperature by 16.4 °C – 18.0 °C for the DNA/DNA duplex, indicating that the pyrene is co-axial stacking with both sides of the duplex, as the stabilization per modification exceeds the effect of placing the pyrene module at one end of a duplex (Table 1). Calculations from "MacroModel" shows that the pyrene module only makes a minor distortion of the double helix  
10 when intercalated into the duplex, having interaction with nucleobases both to the 5' side and to the 3' side of the intercalation site (Figure 3). The stabilization of the duplex by co-axial stacking of the pyrene moiety is not large enough to compensate for the loss in binding affinity due to the reduced number of hydrogen bonds by substitution of G with the pyrene moiety, the modified duplex being less stable than the  
15 unmodified fully complementary by 8.6 °C. The same trend is found for DNA/RNA duplexes although these have lower melting temperatures in general than the corresponding DNA/DNA duplexes. The stabilization of the pyrene moiety is only 8.2 °C for the DNA/RNA duplex when compared with ethylene glycol whereas the stabilization is 16.4 for the DNA/DNA duplex. The pyrene insertion results in an improved  
20 discrimination between ssDNA and ssRNA with 9.0 °C difference in the melting temperatures of their corresponding duplexes.

**Table 2** DNA/DNA and DNA/RNA duplexes where one nucleotides is either an abasic, flexible linker, the pyrene module (5), a deletion or a complementary G.



Entry		DNA $T_m$ (°C)	RNA $T_m$ (°C)	Discrimination $\Delta T_{m, \text{DNA-RNA}}$ (°C)
1	X = 	26.0	25.8	0.2
2	X = 	27.6	26.8	0.8
3	X = <b>5</b>	44.0	35.0	9.0
4	X = - (12-mer)	35.2	29.6	4.6
5	X = G (13-mer)	52.6	47.2	5.4

#### Example 9

#### 5 **1-O-(pyrenylmethyl)glycerol as a bulge.**

Normally the introduction of a bulge into the double helix decreases the melting temperature. This is also observed here (Table 3), but if the pyrene module is built in as the bulge, the melting temperature of the DNA duplex goes up by 3°C. This is in accordance with the observations made by Ossipov et al., finding it necessary to introduce a bulge to prevent a large destabilization of the duplex when introducing a non-Watson-Crick binding intercalator. One pyrene moiety stabilises the duplex by 11.2 °C compared to the flexible ethylene glycol linker indicating that the pyrene moiety is intercalated into the duplex.

15

The difference in the melting temperature between the pyrene modified DNA/DNA duplex and the pyrene modified DNA/RNA duplex is increased to 12.6 °C when inserting one pyrene modification as a bulge. This difference is 7.4 °C larger than in the unmodified duplexes and much larger than the differences between the duplexes containing natural nucleoside or flexible ethylene glycol bulges. This means that the pyrene moiety is selective and only able to stabilize DNA/DNA duplexes and not the DNA/RNA duplex. For the latter duplex it occurs that the duplex have the

20

same melting temperature with the glycerol linker than with the pyrene moiety, indicating that the pyrene does not intercalate into the strands.

Structural calculation of the pyrene modified DNA/DNA structure (figure 4) shows that the pyrene module only makes minor distortion in the duplex, and that the linker introduces enough flexibility in the backbone to have a distance of 3.4 Å between the pyrene moiety and the nucleobases of the same strand. The nucleobases of the opposite strand has a little shorter spacing between the pyrene moiety and the nucleobases than the optimum 3.4 Å.

To investigate the discrimination and stabilization phenomena further, some ODNs with two insertions of the pyrene amidite were prepared, to see if the effects of the pyrene module is additive, and indeed it is. The results (Table 3) show that, depending on the distance between two insertions and their neighboring base pairs, it is possible to stabilize the double inserted pyrene DNA/DNA duplex up to 13.4 °C (6.7 °C per modification) compared to the natural DNA duplex. The destabilization of the pyrene modified DNA/RNA duplex is also somewhat additive, so that the difference in melting temperature between the pyrene modified DNA/DNA duplex and the pyrene modified DNA/RNA duplex is up to 25.8 °C when two pyrene modifications is inserted. For both stabilization and discrimination the best results are obtained, when the insertions are separated by four base pairs. When two insertions of the pyrene amidites are placed next to each other in the ODN there is a decrease in melting temperature of 5.2 °C compared to the unmodified DNA/DNA duplex and a decrease of 8.2 °C compared to the mono modified duplex. It is noteworthy that one basepair between two insertions in the DNA/DNA duplex is sufficient to improve DNA/DNA stabilization and DNA/RNA discrimination, when compared with the duplex with only one insertion.

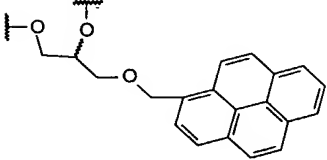

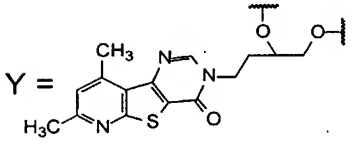

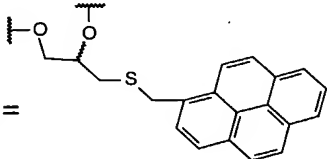

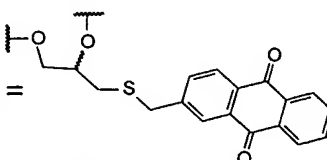

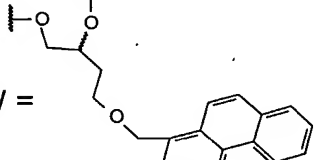

**Table 3 Melting temperatures of oligonucleotides with different substitutions hybridized to either DNA or RNA.**

Example 10**Five different intercalating pseudonucleotides as bulge insertion with increased affinity for the complementary DNA target**

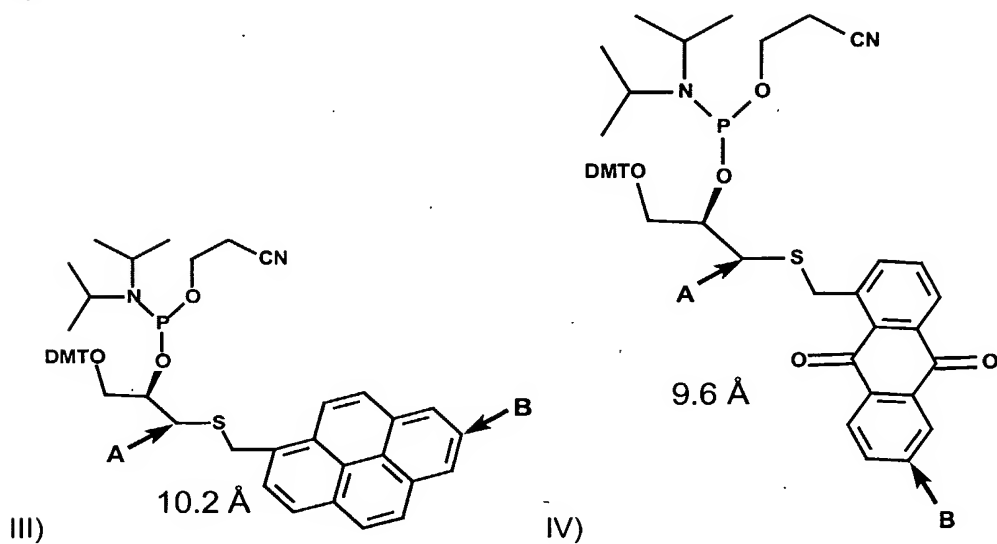
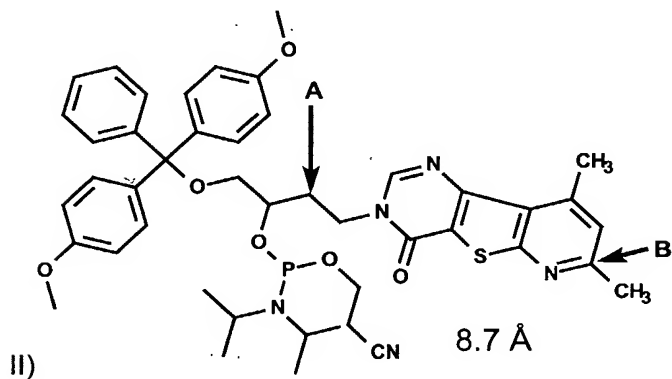
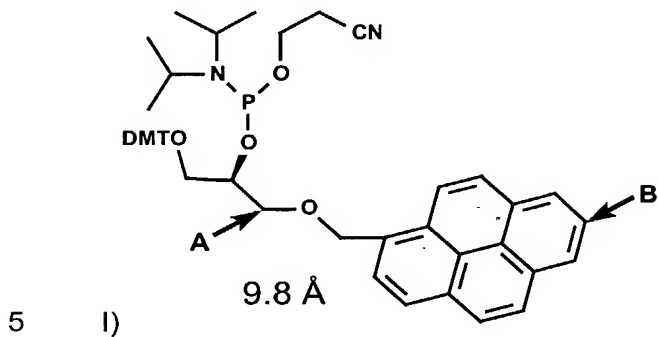
5.

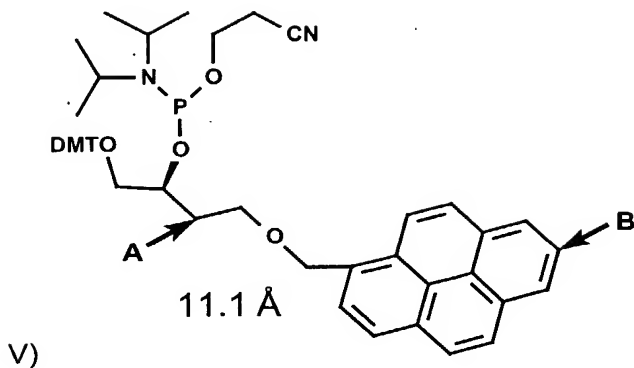
Below is shown an overview of five different intercalating pseudonucleotides inserted in the middle of a DNA oligonucleotide. When hybridised to target said intercalating pseudonucleotides act as bulge insertions. All intercalating pseudonucleotide modified oligonucleotides shown here have an increased affinity for the complementary DNA target compared to the unmodified oligonucleotide:

10

Target DNA strand	5' A-G-C-T-T-G-C-T-T-G-A-G	$T_m$ , DNA
	3' T-C-G-A-A-C-G-A-A-C-T-C	47.4 °C
	3' T-C-G-A-A-C  G-A-A-C-T-C	50.4 °C
	3' T-C-G-A-A-C  G-A-A-C-T-C	50.6 °C
	3' T-C-G-A-A-C  G-A-A-C-T-C	49.4 °C
	3' T-C-G-A-A-C  G-A-A-C-T-C	49.2 °C
	3' T-C-G-A-A-C  G-A-A-C-T-C	48.8 °C

Below is shown the combined length of the linker and intercalator, it is clear that all of the shown examples have nearly the same combined length of intercalator and linker ( $9.9 \pm 1.3 \text{ \AA}$ ).





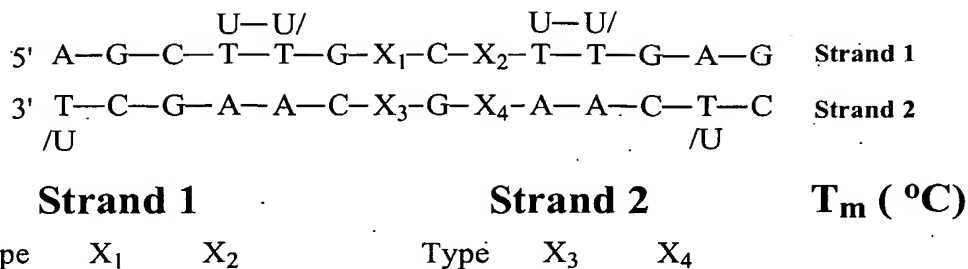
We can from this conclude that intercalating pseudonucleotides are a broad group of compounds that obeys some simple rules regarding the combined length of the intercalator and linker.

#### Example 11

Higher affinity for DNA - lower affinity for RNA.

In this example it is shown that when the target nucleic acid sequence for a probe is DNA, the melting temperature of the hybrid increases by introduction of intercalating pseudonucleotides into the probe – regardless if the probe is DNA or RNA (see Table 4 below). Additionally the affinity for a RNA target is reduced regardless if the probe is DNA or RNA. Hence, intercalator pseudonucleotides can be introduced to oligonucleotides or oligonucleotide analogues giving the oligonucleotide or oligonucleotide analogue increased affinity for DNA and reduced affinity for RNA and RNA-like compounds like LNA, 2'-O-METHYL RNA.





### DNA duplexes and hybrids

DNA	-	-	DNA	-	-	48.4
DNA	5	5	DNA	-	-	51.4
DNA	-	-	DNA	5	5	48.4

### RNA duplexes and hybrids

RNA	-	-	RNA	-	-	57.8
RNA	5	5	RNA	-	-	46.8
RNA	-	-	RNA	5	5	47.2

### DNA/RNA hybrids

DNA	-	-	RNA	-	-	42.2
DNA	5	5	RNA	-	-	34.2
DNA	-	-	RNA	5	5	45.2

5 Table 4: Three different situations. At the top: DNA duplex affinity is increased or unaltered by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strands comprise intercalator pseudonucleotides. In the middle: The RNA duplex is destabilized by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strands comprise intercalator pseudonucleotides. At the bottom: Here it is shown how the hybrid between a DNA and a RNA strand is stabilized by intercalator pseudonucleotides if these are comprised by the

10 RNA strand. Furthermore it is shown than when incorporated into the DNA strand the affinity for RNA is decreased. 5 = amidite 5 from example 1 incorporated into the strand according to the procedure described herein above.

By different relative positioning of the intercalator pseudonucleotides it is possible to gain higher affinity for DNA than shown in the Table above. Examples of this are given in the table in example 9.

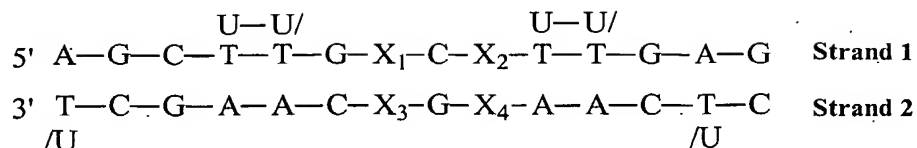
5

#### Example 12

Reduced cross-hybridization .

10 Cross-hybridization between two corresponding oligonucleotides comprising at least one intercalator pseudonucleotide with reduced affinity depending of the relative positioning of the intercalator pseudonucleotides. In the table below it is shown how the melting temperature is decreased if intercalator pseudonucleotides are positioned right opposite each other.

15

**Strand 1****Strand 2****T<sub>m</sub> (°C)****DNA duplexes and hybrids**

DNA	-	-	DNA	-	-	48.4
DNA	5	5	DNA	-	-	51.4
DNA	5	5	DNA	5	5	42.6

**RNA duplexes and hybrids**

RNA	-	-	RNA	-	-	57.8
RNA	5	5	RNA	-	-	46.8
RNA	5	5	RNA	5	5	45.4

**RNA and DNA hybrids**

DNA	-	-	RNA	-	-	42.2
DNA	-	-	RNA	5	5	45.2
DNA	5	5	RNA	5	5	37.8

Table 5: Three different situations. At the top: DNA duplex is stabilized by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strands comprise intercalator pseudonucleotides. If intercalator pseudonucleotides are positioned in relation to each other, so that they are in close vicinity of each other when the oligonucleotides or oligonucleotide analogues are hybridized the melting temperature is decreased compared to when only one strand comprises intercalator pseudonucleotides. In the middle: RNA duplex is destabilized by the presence of intercalator pseudonucleotides in the hybrid compared to the duplex where none of the strand comprise the intercalator pseudonucleotides. At the bottom: Here it is shown how the hybrid between a DNA and a RNA strand is stabilized by intercalator pseudonucleotides if these are comprised in the RNA strand. Further it is shown that if intercalator pseudonucleotides are positioned in relation to

each other, so that they are in close vicinity of each other when the oligonucleotides or oligonucleotide analogues are hybridized the melting temperature is decreased compared to when only the RNA strand comprises intercalator pseudonucleotides. 5 = amidite 5 from example 1 incorporated into the strand according to the procedure described herein above.

#### Example 13

#### 10 **Fluorescence.**

A decreased fluorescence of the mono pyrene modified DNA strands upon binding to the complementary strands, indicates that the pyrene intercalates into the double helix. Double pyrene inserted oligonucleotides gives the same result for all of the different ODNs. This effect is more pronounced when the modified DNA is hybridized with ssDNA than when hybridized with ssRNA (Figures 5 and 6), indicating less intercalation of pyrene into the DNA/RNA Duplexes. This supports the conclusion from the thermal melting experiments about lacking of pyrene intercalation into the bulged DNA/RNA duplexes as deduced from the nearly identical melting temperatures with glycerol and pyrene bulges in the DNA/RNA duplexes (Table 3).

Two pyrene moieties separated by only one nucleotide generates a third peak at 480 nm, due to excimer formation of the pyrene residues. However this band is almost extinguished, when this type of DNA with two insertions with pyrene hybridizes to a complementary DNA strand. This indicates intercalation around an intact base-pair preventing the two pyrene moieties to get into the physical distance of approximately 3.4 nm needed for excimer formation. When a double inserted DNA hybridizes to a complementary RNA the two pyrene moieties are still able to interact since a substantial excimer band is found.

30

#### Example 14

### 3-Exonuclease stability of oligonucleotides or oligonucleotide analogues comprising intercalating pseudonucleotides

#### Materials and Methods:

5

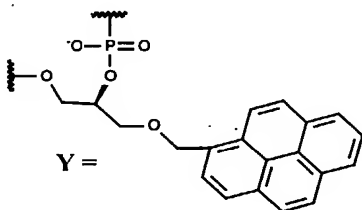
Time course of Snake Venom phosphodiesterase digestion of the DNA reference I, the INA oligo II and a mixture of both I and II. A 1.5  $\mu$ M solution of all the strands was use (1.5  $\mu$ M of each strand in the mixed assay) in 2 mL of buffer (0.1 M Tris-HCl; pH = 8.6; 0.1 M NaCl; 14 mM  $MgCl_2$ ) was digested with 1.2 U Snake Venom phosphodiesterase (30  $\mu$ L of the following buffer solution: 5 mM Tris-HCl; pH = 7.5; 50% glycerol (v/v)] at room temperature.

10

DNA oligo: 3'-TGT CGA GGG CGT CGA

INA oligo: 5'- YAC AGC YTC CCY GCA GCY T

15



#### Results

20

The stability of an INA (Intercalating Pseudonucleotide comprised Nucleic Acid) oligonucleotide toward 3'-exonucleolytic degradation in vitro was evaluated and compared to normal DNA's stability using the Snake Venom phosphodiesterase (SVPDE), Figure 16. It is shown that the reference DNA oligonucleotide, I, is totally digested by SVPDE within 15 min. In contrast the INA oligonucleotide only shows a small hyperchromicity within the first 15 min. and thereafter no significant hyperchromicity is observed. These experiments indicates that the DNA nucleotide in the 3'-end of the INA oligonucleotide is digested by SVPDE, but when the enzyme meets the first intercalating pseudonucleotide it is stalled and unable to digest further.

25

30

The experiment where strand I and II are mixed in the SVPDE assay, giving a hybrid. A slow digestion compared to the reference DNA strand alone is observed. Almost a full degradation of the DNA strand is observed after 60 min. This result indicates that the hybrid, is degraded slower than the single stranded DNA.

5

### Example 15

## **Hairpin shape oligonucleotides comprising intercalating pseudonucleotides for the detection of nucleic acid**

10

### **Introduction**

In this example it is shown how hairpin shaped oligonucleotides comprising intercalating pseudonucleotides (probe I) can be used for the detection of nucleic acids. It is further more shown that using this principle it is possible to detect as low as a 5 nM solution (1 pmol in 200  $\mu$ L) of target nucleic acid. It is also shown that the addition of Hexadecyl trimethyl ammoniumbromide (HTMAB) can enhance the signal sensitivity in a concentration dependent matter.

15

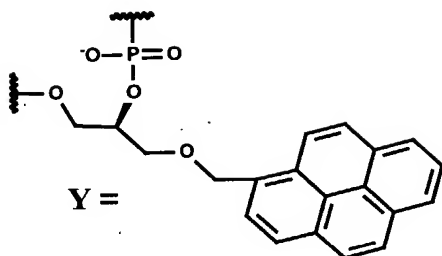
### **Materials and Methods**

20

Below is shown the sequence of the detection probe comprising intercalating pseudonucleotides. The nucleotides which is involved in the hairpin formation is underlined and the nucleotides that are involved in the binding to target is in shown in **bold** letters:

25

Probe I: 5'- CAT CCG YAY **AAG CTT CAA** TCG GAT GGT TCT TCG



In figure FIGURE 17 is shown the secondary structure of the hairpin. The hydrogen bonds of the basepairs in the stem is shown as dots.

- 5 Below is shown the sequence of the target used in these experiments. The nucleotides participating in the binding of the detection probe is shown in **bold** letters:

Target: 3'- ATA GTA TTT ATT **CGA AGT TAG CCT ACC AAG AAG** CCT TTT TTG

- 10 All hybridisation experiments were carried out in a buffer solution containing:

140mM NaCl

10 mM Na<sub>2</sub>HPO<sub>4</sub>•2 H<sub>2</sub>O

1 mM EDTA

- 15 pH = 7.0

The surfactant used in the experiments was HTMAB:

- 20 In Figure **FIGURE 18** is shown a figure that illustrates when the probe binds to its target sequence. It is shown that when the probe is hybridised to the Target, the two pyrene moieties from the intercalating pseudonucleotides are no longer separated by an intact base pair. This makes it possible for them to interact more freely, giving rise to higher excimer fluorescence:

25

In the table below is shown the designed of the experiment:

Wells	1	2	3	4	5	6	7	8
a	H <sub>2</sub> O	buffer	Probe I 100	Probe I 10	Probe I 1 pmol	Probe I 100	Probe I 10	Probe I 1 pmol

			pmol	pmol		pmol + Tar- get 100 pmol	pmol + Tar- get 10 pmol	+ Tar- get 1 pmol
<b>b</b>	H <sub>2</sub> O + sur- factant 10 <sup>-6</sup>	buffer + sufac- tant 10 <sup>-6</sup>	Probe I 100 pmol + sur- factant 10 <sup>-6</sup>	Probe I 10 pmol + sur- factant 10 <sup>-6</sup>	Probe I 1 pmol + sur- factant 10 <sup>-6</sup>	Probe I 100 pmol + Target 100 pmol + sur- factant 10 <sup>-6</sup>	Probe I 10 pmol + Target 10 pmol + sur- factant 10 <sup>-6</sup>	Probe I 1 pmol + Tar- get 1 pmol + sur- factant 10 <sup>-6</sup>
<b>c</b>	H <sub>2</sub> O + sur- factant 10 <sup>-5</sup>	buffer + sufac- tant 10 <sup>-5</sup>	Probe I 100 pmol + sur- factant 10 <sup>-5</sup>	Probe I 10 pmol + sur- factant 10 <sup>-5</sup>	Probe I 1 pmol + sur- factant 10 <sup>-5</sup>	Probe I 100 pmol + Target 100 pmol + sur- factant 10 <sup>-5</sup>	Probe I 10 pmol + Target 10 pmol + sur- factant 10 <sup>-5</sup>	Probe I 1 pmol + Tar- get 1 pmol + sur- factant 10 <sup>-5</sup>
<b>d</b>	H <sub>2</sub> O + sur- factant 10 <sup>-4</sup>	buffer + sufac- tant 10 <sup>-4</sup>	Probe I 100 pmol + sur- factant 10 <sup>-4</sup>	Probe I 10 pmol + sur- factant 10 <sup>-4</sup>	Probe I 1 pmol + sur- factant 10 <sup>-4</sup>	Probe I 100 pmol + Target 100 pmol + sur- factant 10 <sup>-4</sup>	Probe I 10 pmol + Target 10 pmol + sur- factant 10 <sup>-4</sup>	Probe I 1 pmol + Tar- get 1 pmol + sur- factant 10 <sup>-4</sup>

All the probes and targets were annealed in 200  $\mu$ L buffer separately in Eppendorf tubes at 95°C for 2.5 min. and then slowly cooled to room temperature and trans-



ferred to a 96-well black plate from NUNC. The fluorescence was measured on a Wallac Victor<sup>2</sup>, 1420 Multilabel counter, with the following specifications:

Emission filter: F340

5 Excitation filter: 500-10F

Measurement time: 0.1s, 4.0 mm from the bottom of the plate.

CW-lamp energy: 50054, Constant Voltage control.

### Results and Discussion

10 Below is shown the results of the measurement:

Wells	1	2	3	4	5	6	7	8
a	4299	3471	4927	3204	2639	20643	4988	3841
b	5709	3619	9684	4563	3644	18611	3971	4308
c	4021	3119	8429	1879	3456	13833	5337	2202
d	2236	3194	120959	12749	1898	223956	21684	4783

15 As can be seen from comparing a3 with a6, there is a large increase in fluorescence on hybridisation of probe I to a target strand showing proofing the principle in using hairpin-shaped oligonucleotides for detection of nucleic acids sequences. If the background level (a2) is deducted from the measurement, nearly a 12 times increase in fluorescence of probe I upon hybridisation to its target sequence is observed.

20 By comparing a4 with a7 and a5 with a8 it can be seen that it is possible to detect the presence of as low as 10 down to 1 pmol of target nucleic acid.

25 The addition of surfactants on the fluorescence level is also shown. The addition of the HTMAB surfactant increases the fluorescence in some cases more than 100 times (column 6), and hence increases the sensitivity of the detection up to a 100 times.

These results compared with the fact that probe I can be used as a primer in template directed extension reactions makes oligonucleotides or oligonucleotide ana-

logues a very useful tool in e.g. the detection of nucleic acids, for labelling nucleic acids, for the use in extension reactions like ligation and PCR and in real-time quantitative PCR.

5     Example 16

**Control of oligobinding & INA-signal on SAL-chips.**

Method

10

Chip production

- Oligos, 50  $\mu$ M, are spotted dissolved in 400 mM Sodium carbonate buffer, pH 9.
- The chip is instantly placed in humidity-chamber, 37° C for one hour.
- 15     • Oligo binding is effectuated on the surface of the chip by washing with 1%  $\text{NH}_4\text{OH}$ , 5 min.
- Unheated deionised water is used to wash for 2 x 2 min.
- The chips are centrifuged, 600 rpm for 5 min, to remove excess water from the surface.
- 20     • The chip is scanned, or stored refrigerated at 4° C.

SYBR Green II control staining of oligo-binding.

- Deionised water, approximately 90° C is used to wash for 2 x 2 min. Centrifugation, 600 rpm for 5 min follows.
- 25     • 10 000 x dilution of SYBR Green II is added to the chip, apply cover and incubate at ambient temperature for 2-3 min.
- Wash for 1 min, using unheated water and centrifuge the chip to dryness, 600 rpm for 5 min.
- The chip is now ready to be scanned, use Alexa 488 filterset.

30

Results

**Section of chip from OUH, HUMAC, with amino-linker oligos stained with SYBR Green II.**

See Figure **FIGURE 19**

- 5 Spot size: 100  $\mu\text{m}$ . Center-center distance: 175  $\mu\text{m}$

**Evaluation of oligo- and INA-binding on Asper SAL-chips (see Figure **FIGURE 20**).**

10

- INA oligos with signal-modification and aminolinker, presumably bind to SAL chips like normal aminolinker oligos. (see SYBR Green Yes: 1,2,3,4 and 7).
- All signal-INAs fluoresce, when blue or blue-green filtersets are utilized on our ArrayWorx scanner (SYBR Green, No: 1, 2, 3, 4).
- 15 • The position of the signal-modification compared to the oligo-5'-end doesn't seem to be significant. No evident difference is observed between the strength of the signal depending whether the modification is furthest away from the 5'-end (1) or closest to the 5'-end. (4)
- The short 10-mere INAs, without linkers, apparently don't bind to the chip – they lack the linker (see 5). The signal is of the same strength as when clean buffer without oligos is spotted. (See SYBR Green Yes, 5 & 6)
- 20 • The variable background that is shown with different sections of the same chip can be caused by inadequate wash, calibration of scanner, or variation in SAL coating.
- 25 • Observed tendency: Generally the quality of spots, that is shape and signal-homogeneity, seem to be better, when the oligos contain INA modifications (compare 1 and 7, bottom right.)

**Example 17**

30

**Hybridisation properties of DNA-DNA, INA-DNA and INA-INA hybrids at different pH values**

## Introduction

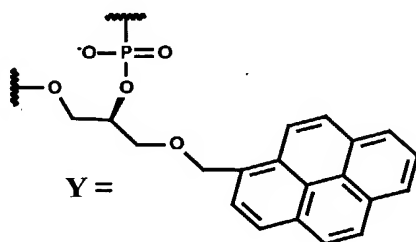
In this example it is shown the hybridisation affinities for DNA-DNA, INA-DNA and INA-INA hybrids at different pH values.

5

## Materials and methods

Hybrids:

10	Hybrid I	5'-CTC AAC CAA GCT 3'-GAG TTG GTT CGA
	Hybrid II	5'-CTC AAC YCA AGC T 3'-GAG TTG GTT CGA
15	Hybrid III	5'-CTC AAC CAA GCT 3'-GAG TTG YGT TCG A
20	Hybrid IV	5'-CTC AAC YCA AGC T 3'-GAG TTG YGT TCG A



25 All hybridisation experiments were carried out with 1.5  $\mu$ M of both target and probe strands in 1 mL of a buffer solution containing:

140mM NaCl

10 mM  $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$

1 mM EDTA

30

The target strands and probes were annealed by mixing them in the above mentioned buffer at 95°C for 3 min. after which they are slowly cooled to room temperature. The melting temperatures of the hybridised probe-target hybrids were found by slowly heating the solution in a quartz cuvette, while simultaneously determining the absorbance. All melting temperatures presented in this example are with an uncertainty of  $\pm 1.0^\circ\text{C}$  as determined by repetitive experiments.

pH was adjusted with a solution of 25%  $\text{NH}_4\text{OH}$  and glacial acetic acid.

## Results and discussion

Hybrid #	pH						
	4.2	5.0	6.1	7.0	8.0	9.0	10.0
I	30.6	43.6	47.8	47.2	49.6	49.6	43.3
II	-	49.7	54.7	54.5	55.7	54.1	51.1
III	-	45.4	51.9	52.5	54.7	52.1	46.4
IV	-	36.1	46.0	46.5	48.5	46.5	40.9

In the table above is shown the results of the melting temperature experiments of hybrid I-IV at different pH values. As can be seen from the table hybrid II and III have higher melting temperatures over the pH range from pH = 5 to 10 than the homologous DNA duplex (hybrid I) and hybrid IV. This shows that it is possible to reduce the cross hybridisation between complementary sequences, when both sequences comprise at least one intercalating pseudonucleotide that are positioned opposite each other when said sequences hybridise. It can also be seen that the melting temperatures of all the hybrids are highest at around pH = 8, and hence in some preferred embodiments it is preferred to hybridise at  $\text{pH} = 8 \pm 2$ . The largest difference in melting temperature between the hybrids II and III comprising one intercalating pseudonucleotide and the hybrid IV comprising two opposite positioned intercalating pseudonucleotides is at pH = 5.0, namely  $13.6^\circ\text{C}$  and  $9.3^\circ\text{C}$  respectively. Hence in a preferred embodiment hybridisations between an oligonucleotide or oligonucleotide analogue comprising at least one intercalating pseudonucleotide and a nucleic acid or nucleic acid analogue is carried out at  $\text{pH} = 5 \pm 1$ .

Example 18

## Preparing a sample for RT-PCR

5

The method of preparing a sample for RT-PCR of a target sequence is depicted in figure 7. The method has the advantage that false positive signals from DNA are largely reduced.

10

A cell sample is provided and the cell walls of the cell are destroyed, thereby releasing DNA and RNA from the cells (figure 7A). Subsequently, an oligonucleotide comprising an intercalator pseudonucleotide, which can hybridise to the target sequence is incubated with the DNA/RNA sample under conditions allowing hybridisation between the oligo and DNA (figure 7B). The sample is then ready to be up-

15 scaled by any standard RT-PCR procedure (figure 7C). Because target DNA present in the sample is blocked by hybridisation to the oligonucleotide, then only RNA may be amplified.

20

Alternatively, after destroying the cell walls, RNA may be purified by any standard method for example by extraction and precipitation (figure 7D). Usually, the purified RNA will comprise small amounts of DNA contamination. Hence, an oligonucleotide comprising an intercalator pseudonucleotide, which can hybridise to the target sequence is incubated with the RNA sample under conditions allowing hybridisation between the oligo and DNA (figure 7E). The sample is then ready to be up-

25 scaled by any standard RT-PCR procedure (figure 7F). Because target DNA contamination present in the sample is blocked by hybridisation to the oligonucleotide, then only RNA may be amplified.

30

Example 19

## Preparing a sample for RT-PCR

The method of preparing a sample for RT-PCR of a target sequence is depicted in figure 8. The method has the advantage that false positive signals from DNA are largely reduced.

- 5 A cell sample is provided and the cell walls are destroyed, thereby DNA and RNA is released. RNA may be purified by any standard method from the sample (figure 8B), however it is also possible to perform the subsequent steps on the DNA/RNA sample.
- 10 The sample is incubated with beads linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 8C), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. After hybridisation the sample is filtered to remove the beads together with bound target DNA from the sample (figure 8D).
- 15 The sample is ready for RT-PCR (figure 8E). Because the sequence specific target DNA has been removed from the sample, the risk of false positives of the RT-PCR due to DNA contamination is largely reduced.
- 20 Alternatively, after sample preparation, the sample is incubated with a solid support linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 9B), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. After hybridisation the solid support is removed from the sample together with bound target DNA. The sample may once
- 25 again be incubated with a solid support linked to an oligonucleotide comprising an intercalator pseudonucleotide to remove traces of sequence specific DNA still left in the sample. The solid support is removed from the sample after hybridisation to sequence specific DNA (figure 9C).
- 30 The sample is then ready for RT-PCR.

#### Example 20

Purification of sequence specific DNA

The purification of sequence specific DNA is illustrated in figure 9 and 10.

5 A cell sample is treated with GnSCN thereby releasing nucleic acids. The sample is incubated with beads linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 10A), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. The sample is filtrated and washed to remove non-bound nucleic acids (figure 10B). The beads are subjected to heating and filtration, releasing pure, sequence specific DNA largely free of sequence specific RNA (figure 10C).

15 Alternatively, the nucleic acid sample is incubated with a solid support linked to an oligonucleotide comprising an intercalator pseudonucleotide (Figure 11B), which can hybridise to the target sequence under conditions allowing hybridisation between the oligo and DNA. The solid support is separated from the rest of the sample and subjected to heating, which releases the sequence specific DNA (figure 11C). The sequence specific DNA will be largely free of sequence specific RNA and is ready for diagnosis, PCR or other purposes.

20 Example 21

Detection of target DNA

25 Oligonucleotides comprising pyrene pseudonucleotides are linked to a chip. The oligonucleotides are designed so that a part of it may hybridise to a specific target DNA and so that the oligonucleotide may also self-hybridise. When the oligonucleotide is hybridised to itself, 3 pairs of pyrene pseudonucleotides are facing each other, and accordingly the melting temperature of a DNA/oligo hybrid is higher than the melting temperature of the selfhybrid. Furthermore, the oligonucleotide comprises two pyrenes capable of forming an excimer, only when the probe is not hybridised to itself (figure 12 and figure 13A).



Different oligonucleotides recognising different target DNAs may be added to various defined regions of the chip. In the present example 2 different oligonucleotides are linked to spot 1 and spot 2, respectively.

- 5 A crude mixture of DNA fragments containing the target DNA is added to the chip at a temperature where the oligonucleotide can not selfanneal.

After an annealing step and a washing step, the temperature is lowered to allow self hybridisation of probes. Excimer formation is used to detect the presence of target  
10 DNA as well as the amount of target DNA hybridised. The procedure is outlined in figure 12.

Alternatively, the oligonucleotide may be designed so that it comprises a fluorophore and a quencher, wherein the fluorophore signal may only be quenched by the  
15 quencher when the oligonucleotide is self-hybridised (figure 13B).

It is also possible to use two oligonucleotides which each comprises 3 pyrenes pseudonucleotides that are facing each other when the oligonucleotides are hybridised. The oligonucleotides also contains a fluorophore and a quencher each, positioned so that the fluorophore signals may only be quenched by the quencher when  
20 the oligonucleotides are hybridised (figure 13C).

### Example 22

#### 25 **Exciplex fluorescence from intercalating pseudonucleotides**

##### **Introduction**

In this example is shown some exciplex fluorescence emission between two intercalating pseudonucleotides.  
30

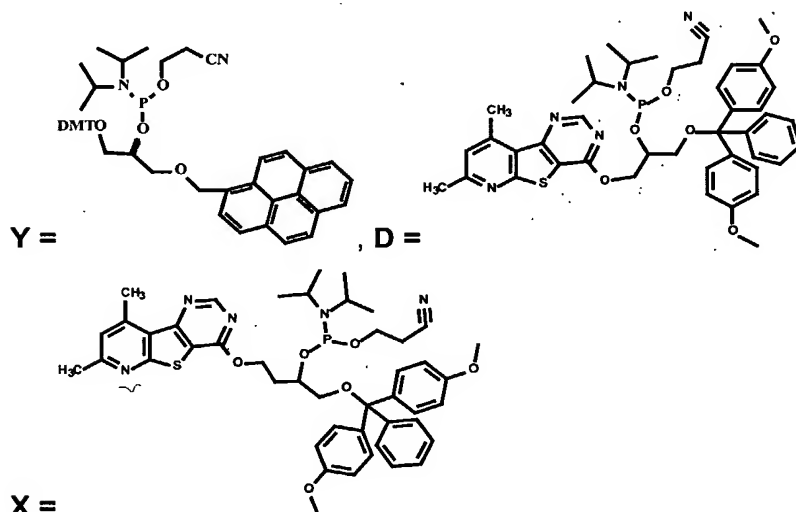
##### **Materials, Methods and Results**

Three oligonucleotides comprising two different intercalating pseudonucleotides were synthesized using standard procedures:

Sequence 1: 5'-CTCAAYGDCAAGCT

5 Sequence 2: 5'-CTCAAGYDCAAGCT

Sequence 3: 5'-CTCAAGYXCAAGCT



10 After purification by HPLC the oligonucleotides comprising intercalating pseudonucleotides was dissolved in a buffer solution containing:

140mM NaCl

10 mM Na<sub>2</sub>HPO<sub>4</sub>•2 H<sub>2</sub>O

15 1 mM EDTA

pH = 7.0

and all fluorescence experiments were carried out in this buffer. Excitation was done at 343 nm on a Perkin Elmer MPF-3 spectrophotometer with at xenon 150 power supply.

20 As seen from Figures FIGURE 21 and FIGURE 22, the exciplex to monomer fluorescence ratio was higher when the to intercalating pseudonucleotides were positioned as neighbours (Figure FIGURE 21) than when placed as next-nearest neighbours (Figure FIGURE 22) - the exciplex transition was however clearly observed in both cases. Similar result was obtained with the amidite X.

25

As a conclusion an exciplex between two intercalating pseudonucleotides can be observed for both neighbouring and next-nearest neighbouring intercalating pseudonucleotides, when said pseudonucleotides are positioned internally in an oligonucleotide (See Figure FIGURE 23).

### Example 23

#### **PCR with oligonucleotide primers comprising intercalator pseudionucleotides**

35 cycles of gradient PCR (94°C, 30 sec; gradient annealing temp, 45 sec; 72°C, 60 sec.) were performed with diluted plasmid template in a standard PCR-buffer (1.5 mM MgCl<sub>2</sub>; 50 mM KCl; 10 mM Tris-HCl; 0.1% Triton X-100, 200  $\mu$ M of each dNTP, 5 pmol of each primer) in a final volume of 25  $\mu$ l. PCR products were separated in a 0.7% agarose gel in 1xTBE buffer and visualized by EtBr staining. Temperatures on the figure 24 denote the annealing temperature in each well.

Primer designs (upstream and downstream, respectively)

a01	5'-	AAGCTTCAATCGGATGGTTCTTCG
a02	5'-	YAAGCTTCAATCGGATGGTTCTTCG
a03	5'-	YCYATCCGAAAGCTTCAATCGGATGGTTCTTCG
a05	5'-	CYAYTCCGAAAGCTTCAATCGGATGGTTCTTCG
b01	5'-	CACAAGAGCTGACCCAATGGTTGC
b02	5'-	YCACAAGAGCTGACCCAATGGTTGC
b03	5'-	YTYGGGTCACACAAGAGCTGACCCAATGGTTGC
b05	5'-	TYGYGGTCACACAAGAGCTGACCCAATGGTTGC

Primers 03 and 05 are able to form hairpin loops when not hybridized with target as exemplified below by the a05 primer:

Primer alone	TCAA
T	TCGGA T GGTTCTTCG-3'

C AGCCTYAY

GAA

C-5'

Primer 5'- CYAYTCCGA

5

AAGCTTCAATCGGATGGTTCTTCG

TTCGAAGTTAGCCTACCAAGAAGC

Target:

5'- TA

CT

10

Conclusion

15

As can be observed from the picture of the gel, the addition of one single end-positioned intercalator pseudonucleotide in the linear primers a02/b02 compared to the DNA control primers raises the effective melting temperature significantly.

From the amplification products of bands for the 03 and 05 primers it primers with beacon design primes PCR in a highly efficient manner.

20

Example 24**INA-oligo binding to DNA target****Results and discussion**Binding requires target-specificity and occurs spontaneously.

25

A series of INA-oligonucleotides were designed and tested for their abilities to spontaneously bind an 80 bp complementary target DNA sequence (Fig. 25).

Reactions were carried out by incubation of the double-stranded target DNA with an excess of P32-labelled INA-oligos (IOs) in a sodium-phosphate buffer containing 120 mM Sodium chloride at 37 °C for 1-3 hours. Results were then evaluated by electrophoretic mobility shift analysis and phosphor-imaging of the labelled IOs.

30

Figure 26 shows that all three IOs tested (IO 1-1, IO 1-2, IO 1-3) bound the target DNA. The relative amounts of bound IOs were determined by volume analysis of the retarded bands using the ImageQuant software. As the numbers at the bottom of the figure indicates the IOs showed different affinities for the target. The IO 1-3 clearly

had an advantage in binding the target and was therefore chosen for further analysis.

Evidence for the specificity of the observed binding was next ascertained. First P32 labelled IO 1-3 was incubated with either strand of the DNA target alone. As expected based on sequence complementarity IO 1-3 specifically bound the sense strand of the DNA target (Fig. 27, lane 1-3). Binding to the double stranded DNA target was then assayed at increasing concentration of P32 labelled IO 1-3 and compared to binding to an unrelated 60 bp target DNA sequence (compare lanes 4-5 with 7-8). Clearly, target binding by the IO required target complementarity as no binding was observed with the sequence-unrelated target DNA. To verify the position of the observed retardation a fraction of the target DNAs were P32 labelled and their retardation assayed in parallel (lanes 6 and 9).

IO pairing does not inhibit spontaneous binding and gives variable target-affinities by differential positioning of the intercalating units.

The observed target strand specificity of the IOs (ie. IO 1-3 specifically binding the sense strand of the target DNA, see above) suggested that the antisense strand of the target DNA may be free to be simultaneously attacked by a different IO. To explore this possibility IO 1-3 was annealed to three different, complementary IOs. As shown in figure 28 the pairing of the IOs still rendered the P32 labelled IO 1-3 capable of spontaneously binding the target DNA, albeit with different affinity depending on the positioning of the intercalating units in the pairing IO. As the IO 1-3 / IO 5 pair gave the best target-binding this pair was chosen for further testing. To investigate whether pairing affects the efficiency of spontaneous target binding, binding of IO 1-3 was assayed with and without previous pairing to IO 5. Figure 29 shows that pairing did not affect the spontaneous binding of IO 1-3 to the target DNA.

#### **Nuclear factors aid IO target binding and favours paired IOs**

RecA/Rad51 assisted joint molecule formation between DNA targets and small RNA-DNA oligonucleotides have previously been reported (Gamper 2000, Yoon 2002). The IO readily bound the target unassisted and thus hold promise as *bona fide* agents for DNA targeting for therapeutic purposes. It therefore was of great importance to clarify how this binding would proceed in a nuclear environment. To address this subject we employed nuclear extracts prepared from human cell culture.

As shown in figure 30, when reactions were carried out in the presence of nuclear extract retardation of IO 1-3 was at least 3-5 fold increased (calculations not shown), and only occurred in the presence of the specific target. Moreover, the degree of binding was dependent on the amount of nuclear protein added, and as such increased until a certain amount of protein was added. It then decreased as would be expected based on similar analysis with addition of protein involved in DNA repair processes. It is generally assumed that this process involves a D-loop formation of the targeted DNA, leaving both strands open for attack by matching oligonucleotides. It was therefore interesting to observe that upon addition of nuclear extract pairing IO's did indeed enhance the binding of the P32-labelled IO 1-3 to the DNA target (Figure 31).

## Materials and Methods

**Oligo-synthesis:** all oligos were prepared by standard procedures.

**Radioactive labelling of oligos:** oligos were endlabelled by incubation with polynucleotide kinase and  $\gamma$ -P<sup>32</sup> ATP. Labelled oligoes were purified by the Mermaid kit procedure.

**Nuclear extracts:** HT-29 extracts were prepared from pre-confluent HT-29 colon-cancer cells by the NUN extraction procedure. HeLa nuclear extracts were obtained from (a company in Belgium).

**Electrophoretic mobility assay:** Reactions were carried out in 20 mM sodium-phosphate buffer pH 8.0 containing 120 mM NaCl, 1 mM DTE. Upon incubation at 37°C reactions were snap-frozen in liquid N<sub>2</sub> and stored at -80°C or applied directly to electrophoresis on 7 or 10 % polyacrylamide gel cast in ½ x TBE, at 300 V for 2-4 h, at 4°C.

Reactions containing nuclear extracts additionally contained 0.22M Urea, a total of 200mM NaCl, 0.22 % NP-40, 5.52 HEPES, 5 mM MgCl<sub>2</sub> and 2 mM ATP. These reactions were incubated at 37°C for 10 min. upon which 1.175 µl 10% SDS and 37.5 µg Proteinase K was added and incubation reassumed for another 60 min.

For comparative purposes equal CPM of individual IOs were added to reactions.

**Visualisation:** EMSA results were evaluated using a STORM phosphorimager and the ImageQuant software.

### Example 25

5

## **LNA + INA: Making locked nucleic acid hairpins accessible to targeting by insertion of intercalating nucleic acid monomers**

### 10 **INTRODUCTION**

Hairpin structures are a common feature of single-stranded DNA and RNA sequences. This type of secondary structures can make target sequences inaccessible to intermolecular Watson-Crick base pairing (i). There is a need to find new techniques to alleviate this problem. The ones previously reported are using fragmentation of the nucleic acid sequence close to the target (ii) or replacing natural 2'-deoxycytidine with *N*<sup>4</sup>-ethyl-2'-deoxycytidine which forms base pairs with 2'-deoxyguanosine having reduced stability as compared with natural base pairs (iii). A variety of modified oligonucleotides have been developed during the last two decades in order to develop potential gene inhibitors which possess an enhanced stability towards cellular nucleases, the ability to penetrate the cell membrane and an efficient hybridisation to the target RNA/DNA. If the modified oligonucleotides have efficient hybridisation properties, they are also expected to form secondary structures which can make a large number of sequences inaccessible, but to our knowledge no attempts have been done on modified oligonucleotides to overcome this problem.

LNA oligonucleotides are oligonucleotides containing a conformationally restricted monomer with a 2'-O, 4'-C-methylene bridge (Fig. 1) and they have shown helical thermal stability when hybridised to either complementary DNA or RNA when compared with unmodified duplexes. Due to their hybridisation efficiencies they are also expected to form extremely stable hairpin structures. It is challenging also to make LNA hairpin structures accessible to targeting as LNA seems to be the most promising antisense candidate among modified oligonucleotides.

INAs (Intercalating Nucleic Acids, Fig. 1) composed by insertions of intercalating pseudonucleotides into DNA are strongly discriminating between DNA

over RNA when hybridising with them (<sup>iv</sup>). Properly designed INAs gives more stable INA/DNA duplexes than its DNA/DNA counterparts whereas the opposite is found when INAs are hybridising to RNA which results in less stable duplexes than the corresponding RNA/RNA duplexes. In this paper it is shown that this property can be used to make LNA hairpins more accessible to targeting of DNA by inserting INA monomers into the stem of the hairpin.

## **MATERIALS AND METHODS**

### **Synthesis of DMT protected LNA and INA phosphoramidites**

The phosphoramidite of LNA and INA, respectively, were prepared as previously described (4a,8a).

### **ODN, LNA and INA synthesis, purification and measurement of melting temperatures.**

The ODN, LNA and INA synthesis was carried out on an Expedite™ 8909 Nucleic Acid Synthesis System from Applied Biosystems. The LNA and INA amidite was dissolved in a 1:1 mixture of dry acetonitrile and dry dichloromethane, as a 0.1 M solution, and inserted into the growing oligonucleotides chain using same conditions as for normal nucleotide couplings (2 min coupling). The coupling efficiency of the modified nucleotide was > 99%. The ODNs, LNAs and INAs were synthesised with DMT on and purified on a Waters Delta Prep 3000 HPLC with a Waters 600E controller and a Waters 484 detector on a Hamilton PRP-1 column. Buffer A: 950 ml of 0.1 M NH<sub>4</sub>HCO<sub>3</sub> and 50 ml MeCN, pH 9.0; buffer B: 250 ml of 0.1 M NH<sub>4</sub>HCO<sub>3</sub> and 750 ml MeCN, pH 9.0. Gradients: 5 min 100% A, linear gradient to 100% B in 40 min, 5 min with 100% B, linear gradient to 100% A in 1 min and then 100% A in 29 min (product peak at ~ 37 min). The ODNs, LNAs and INAs were DMT deprotected in 925 µl of H<sub>2</sub>O and 75 µl CH<sub>3</sub>COOH and purified by HPLC, again using the same column, buffer system and gradients (product peak at ~ 26 min). To get rid of the salts, the ODNs, LNAs and INAs were redissolved in 1 ml of water and concentrated *in vacuo* three times.

All ODNs, LNAs and INAs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. The transition state analyses were carried out on a Perkin Elmer UV/VIS spectrometer Lambda 2 with a PTP-6 temperature programmer using PETEMP rev. 5.1 software and PECSS software package v. 4.3. All ODNs were measured in a 120 mM NaCl,



10 mM, sodium phosphate, 1 mM EDTA, pH 7.0, 3.0  $\mu$ M each strand. All melting temperatures are with an uncertainty  $\pm 0.5^\circ\text{C}$  as determined by repetitive experiments.

## 5 RESULTS AND DISCUSSION

### Duplexes

NMR has been used to determine the structure of DNA/LNA duplexes and it was found that only one LNA monomer in the duplex was sufficient to induce a change in the sugar conformation of the flanking nucleotids from a north conformation typically found in B-type DNA/DNA duplexes to a south conformation, the latter being the one typically found in the sugar parts of A-type RNA/RNA duplexes (<sup>v</sup>). It was therefore found interesting to investigate systematic insertions of the INA monomer **P** (Fig. 32) at all possible sites of this duplex (Figure 33). The oligo **I** without any LNA monomers was used as a reference target for the hybridisations with the probes 2-12 (Figure 33) having insertions of **P** at all possible positions, except at the 3'-end. The oligo **I** had in all cases increased duplex stabilities when compared with the unmodified probe 1. In fact, probe 3 gave a remarkably stable duplex with an increase of 10.1  $^\circ\text{C}$  in the thermal melting temperature. This oligo has the INA monomer **P** inserted in an AT region and when compared with the other probes, this seems generally preferable.

For the oligo **II** it was observed that insertions of the INA monomer **P** into regions of its duplexes away from the LNA monomer increased the duplex stability when compared with the unmodified probe 1. In fact the stabilisations were nearly identical with those observed for the oligo **I** and this confirms that regions away from the LNA monomer have still a B-type structure. Only when the insertion of **P** was done into the complementary oligo close to the LNA monomer (Figure 33, entries 2-5), differences could be observed in hybridisation efficiencies between the two oligos upon hybridisation to their complementary sequences. This could be ascribed to a conformational change of the sugar part of the neighbouring nucleotides and this was reflected by a decrease in thermal melting temperatures. The major differences were found for the duplexes with neighbouring insertions to the LNA monomer (Figure 33, entries 2 and 3) whereas minor differences were found for duplexes with next neighbouring insertions (Figure 33, entries 4 and 5).

Oligo **III** with three evenly placed LNA monomers is supposed to induce A type duplex structure in most parts of the duplex formed on its hybridisation

with its complementary sequence. This is deduced by comparing  $\Delta T_{ms}$  for the oligo III with those for the oligo I. The conclusion is in agreement with NMR structure determination on a similar duplex with three LNA monomers (<sup>v</sup>). Comparable stabilisations were only found for these two oligos when the INA monomer **P** was inserted close to the ends of their respective duplexes and thus confirming B-type duplex at the ends (Figure 33, entries 10 and 11).

The study on the oligos I-III demonstrates INA monomer **P** insertions as a versatile tool of distinguishing A and B type duplex regions when a modified nucleotide induces an A type duplex structure into a region of a B type duplex.

### Hairpins

On heating the hairpin forming oligo T<sub>4</sub>-DNA (see Figure 34 for sequence key) alone in a thermal melting experiment a clear transition is observed at 37.2 °C (Figure 2A and Figure 34) which is ascribed to opening of the hairpin to an ssDNA. In a similar experiment with T<sub>4</sub>-LNA (Figure 34) a higher transition temperature is to be expected for opening of the corresponding hairpin due to higher stability of the stem which is deduced from the reported higher stabilities of LNA/DNA duplexes. To test this hypothesis T<sub>4</sub>-LNA analogous to T<sub>4</sub>-DNA was synthesised with five nucleotides in the stem being replaced with the corresponding LNA monomers (T<sup>L</sup> and MeC<sup>L</sup>, respectively). For this modified hairpin only an incipient transition from hairpin to ssLNA could be observed above 80 °C (Fig. 35A). An equimolar mixture of T<sub>4</sub>-DNA and A<sub>4</sub>-DNA gives a transition at the same temperature as the one observed for T<sub>4</sub>-DNA alone, but the increase of optical density (hyperchromacia) is much stronger for the transition of the A<sub>4</sub>-DNA/T<sub>4</sub>-DNA mixture. The increase in hyperchromacia of the mixture is not due to an additional transition of an A<sub>4</sub>-DNA hairpin because this oligo alone has no transition above 20 °C which is in agreement with earlier reports that adenine compared to thymine in the loop destabilises a hairpin (<sup>vi</sup>). The increased hyperchromacia of the mixture is therefore best explained by the melting of an A<sub>4</sub>-DNA/T<sub>4</sub>-DNA duplex although it is impossible to estimate the ratio of distribution of T<sub>4</sub>-DNA between its hairpin structure and its duplex structure with A<sub>4</sub>-DNA.

Due to the stability of the T<sub>4</sub>-LNA hairpin and the instability of the A<sub>4</sub>-DNA hairpin no transitions were expected to be found in the temperature range 20–80 °C for a mixture of these two oligos. It was therefore puzzling to find a transition at 37.3 °C with a rather low hyperchromacia. The extra transition for the A<sub>4</sub>-DNA/T<sub>4</sub>-LNA mixture is best understood by comparison with the properties of palin-

dromic sequences which have been extensively studied by NMR. For example, it was shown that the self complementary sequence 5'-CGCGTTAACGCG formed a duplex at lower temperatures with a transition to a hairpin at 33 °C at 0.3 mM and again a transition to random coil at 48 °C <sup>(vii)</sup>. As depicted in Figure 36, Scheme 1

5 our system is nearly the same, except that the hairpin forming oligo (T<sub>4</sub>-LNA) does not have a self complimentary sequence in the loop region and needs another oligo to form the duplex. In our case the required oligo (A<sub>4</sub>-DNA) does not form a hairpin at ambient temperatures and is therefore not introducing any complications for the interpretation of the melting of the A<sub>4</sub>-DNA/T<sub>4</sub>-LNA mixture. By comparison with the

10 nature of the palindromic sequences the melting at 37.3 °C of the A<sub>4</sub>-DNA and T<sub>4</sub>-LNA mixture is best explained by a transition from DNA/LNA duplex to a mixture of A<sub>4</sub>-DNA and T<sub>4</sub>-LNA hairpin. For palindromic oligos it has been suggested that the transition from duplex to hairpin takes place through formation of a cruciform structure formed after creation of an initial bulge in the center of the duplex upon melting

15 <sup>(viii)</sup>. Once the cruciform is formed little energy is needed to propagate the mobile junction formed and to complete the separation of the two hairpins. We can argue for a similar mechanism in our case and also for the same type of mechanism operating in the opposite direction, because identical melting curves for up and down temperature modes were obtained. This could implicate that we have found an ex-

20 ample of strand invasion into an extremely stable LNA hairpin.

For the 5'-CGCGTTAACGCG sequence, hairpin structures were always observed by NMR at lower temperatures and complete conversion from hairpin to duplex was never observed <sup>(vii)</sup> which may indicate quenching of the equilibrium at temperatures lower than the transition temperature. Also in our case a rather

25 low hyperchromacia for the transition seems to indicate that the conversion from a mixture of A<sub>4</sub>-DNA and T<sub>4</sub>-LNA hairpin to DNA/LNA duplex is incomplete. This implies a more complete transition from hairpin to duplex if the melting temperature is higher and closer to the melting of the LNA hairpin. This is indeed what we found when a pyrene pseudonucleotide is inserted in the middle of the A<sub>4</sub> region in the A<sub>4</sub>-

30 DNA (Figure 34). It has previously been found that a single INA insertion in an A/T region of a duplex causes a significant increase in the melting temperature. This is also observed here with a melting of 44.7 °C, and furthermore, a significant increase in the hyperchromacia is observed when compared with the DNA/LNA duplex from A<sub>4</sub>-DNA (Figure 2A and 2D). The higher hyperchromacia in this case indicates that

the A<sub>2</sub>PA<sub>2</sub>-DNA/T<sub>4</sub>-LNA duplex has a better ability to be formed in the transition from the LNA hairpin.

From the finding above that pyrene insertions opposite to the LNA monomer lower the melting temperatures of LNA containing duplexes, we tested that proper insertions in the stem of a T<sub>4</sub>-LNA hairpin could reduce its stability and make it prone to targeting to A<sub>4</sub>-DNA. As the transition temperature of T<sub>4</sub>-LNA is too high to be determined, it was promising to observe a thermal transition for the mono pyrene inserted oligos P<sup>2</sup>-LNA and P<sup>5</sup>-LNA at 81.1 °C and 71.4 °C, respectively, and for the double pyrene inserted oligo P-P-LNA at 69.1 °C though it has to be admitted that the hyperchromacia was extremely low for the three transitions. This means that one shall be very cautious about the interpretation and this is symbolised by using parentheses for these transitions in Figure 34. Irrespectively whether the transitions are due to opening of hairpins or to undefined duplex meltings, we took it as evidence for assuming that these pyrene inserted oligos could be more accessible for duplex formation with their complementary ssDNA targets.

When the oligo P<sup>2</sup>-LNA with the INA monomer **P** was inserted after the first nucleotide in the stem, a considerably stronger hyperchromacia was observed on melting of the duplex with A<sub>4</sub>-DNA (Fig. 35B) than for T<sub>4</sub>-LNA with A<sub>4</sub>-DNA without any insertion of **P** (Fig. 35A). The hyperchromacia is approximately half of the one observed for the T<sub>4</sub>-DNA/A<sub>4</sub>-DNA duplex which is shown in Figs 2A-C as a reference. An increase in the transition temperature is also observed as should be expected because of the stabilising effect of **P** on hybridisation to a DNA. For both the transition temperature and hyperchromacia a similar result is found for the oligo P<sup>5</sup>-LNA with a **P** insertion close to the loop of its corresponding hairpin form (Fig. 35C). With two **P** insertions in the stem region the resulting oligo P-P-LNA shows an even higher transition temperature for its corresponding duplex with A<sub>4</sub>-DNA, but more strikingly, the hyperchromacia is nearly the same as the one for the unmodified duplex. This is a clear demonstration that **P** insertions into LNA with secondary structures can make this special type of LNA more accessible to targeting and at the same time increase the duplex stability with the target, the latter being deduced from higher transition temperatures for the LNA probes with **P** insertions. It was attempted further to stabilise the duplexes with the LNA probes by inserting **P** into the target in the region corresponding to the loop in the probes. As seen from Figure 34, the oligo A<sub>2</sub>PA<sub>2</sub> showed even higher transition temperatures and again the highest one was found for two **P** insertions in the LNA probe (P-P-LNA). Stabilising the du-

plexes by extra insertions in the target also improved the hyperchromasia as it is seen for the A<sub>2</sub>PA<sub>2</sub> oligo (Fig. 35D). The highest melting temperature was found for (APA)<sub>2</sub>-DNA when forming a duplex with P-P-LNA. In this case the melting temperature is considerably higher (83.3 °C) than the transition temperature (69.1 °C) measured for what is most likely the P-P-LNA hairpin.

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### Example 26

#### Preparation of an intercalator pseudo nucleotide

The example describes preparation and use of *N*-(pyrene-1-ylmethyl)-(3*R*,4*R*)-4-(hydroxymethyl)pyrrolidin-3-ol (**4**) in the synthesis of several INAs and investigated the hybridisation affinity of INA/DNA, INA/RNA duplexes and DNA TWJ region. When the *N*-(pyren-1-ylmethyl)azasugar was inserted as a bulge good discrimination between stabilities of INA/DNA and INA/RNA duplexes and the increased stability of a DNA three-way junction were observed.

The synthesis of 1'-aza pyrenemethyl pseudonucleoside **4** started from enantiomerically pure 1-aza analogues of 2-deoxy-D-hexofuranose **1** or 2-deoxy-D-ribofuranose **2**.<sup>13</sup> Pyrene substrates having chloromethyl and carbaldehyde functionalities that could be coupled with the secondary amines **1** and **2** were used (Fig. 37).

The DMT protected phosphoramidite **6** is required for the oligonucleotide synthesis. The primary alcohol **4** was treated with an excess of DMTCl in pyridine with further purification on a silica gel column to give compound **5** in 61% yield. The synthesis of the final phosphoramidite by treatment with 2-cyanoethyl-*N,N*-isopropylchlorophosphoramidite in the presence of the excess of Hunig's base<sup>12</sup> failed. To obtain the required phosphoramidite **6** we used an alternative method with 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphane and *N,N*-diisopropylammonium tetrazolide.<sup>15</sup> The compound **6** was obtained in 57% yield.

The phosphoramidite **6** was incorporated into different oligonucleotide sequences to give INAs on an automated solid phase DNA synthesizer using an increased coupling time (24 min) and repeating the cycle twice. The coupling efficiencies for the pyrene azasugar derivative **6** were approximately 80-85% compared to approximately 99% for commercial phosphoramidites (2 min coupling).

The synthesised INAs were used in the hybridisation studies of INA/DNA and INA/RNA duplexes (Fig. 38) and INA/DNA three way junction (TWJ) (Fig. 39).

INA with incorporation of the *N*-(pyren-1-ylmethyl)azasugar as the bulge resulted in lowering of the melting temperature with 1.2 °C per modification towards ssDNA (Fig. 15). The corresponding reference duplex in entry **B** containing a bulging de-

oxynucleotide (dG) had a considerably lower  $T_m = 32.2\text{ }^{\circ}\text{C}$  ( $\Delta T_m = -10.8\text{ }^{\circ}\text{C}$ ). For the INA/RNA duplexes the pyrene containing sequence **C** and the reference **B** decreased the stability of the INA/RNA duplex with  $10\text{ }^{\circ}\text{C}$  and  $9.6\text{ }^{\circ}\text{C}$ , respectively; compared to the perfectly matched duplex (entry **A**). Consequently, INA with pyrene azasugar incorporated as the bulge has better hybridisation affinity towards the complementary ssDNA than towards ssRNA. The differences in melting temperatures for ssDNA and ssRNA seems to be additive with respect to the number of pyrene moieties in the targeting ODN. These results are also in agreement with other investigations where 1-O-(1-pyrenylmethyl)glycerol was inserted twice as bulges. A larger discrimination up to  $25.8\text{ }^{\circ}\text{C}$  between INA/DNA and INA/RNA was then observed.<sup>8</sup> In that case the INA/DNA structure is stabilised compared to the wild type duplex in contrast to our case where a slight decrease is observed for  $T_m$ . The flexibility of the bulge may be an important factor to obtain both duplex stabilization and discrimination. The synthesis of different linkers and planar aromatic moieties is also in progress. The RNA/DNA discrimination displayed may be applied for purification or detection of DNA targets in a mixture with the very same sequences of RNA.

DNA three way junction (TWJ) composed of two arms linked to a stem (Fig. 39), was observed to lead to a considerable stabilisation when the pyrene azasugar intercalator was inserted in the INA (**F3**) compared to the ODN having dA at the same position (**F2**) or without an insertion in the ODN (entry **F1**). To be sure that hybridization in the arms is important for the stability of the complex; we prepared ODNs with mismatches in either arm of TWJ (entry **E2** and **E3**). In both cases it resulted in a large lowering of the hybridisation affinity.

## Experimental

### General

NMR spectra were recorded on a Bruker AC-300 FT NMR spectrometer at 300 MHz for  $^1\text{H}$  NMR and at 75.5 MHz for  $^{13}\text{C}$  NMR. Internal standards used in  $^1\text{H}$  NMR spectra were TMS ( $\delta$  0.00) for  $\text{CDCl}_3$ ,  $\text{CD}_3\text{OD}$ ; in  $^{13}\text{C}$  NMR were  $\text{CDCl}_3$  ( $\delta$  77.0),  $\text{CD}_3\text{OD}$  ( $\delta$  49.0). Accurate ion mass determination was performed on a Kraton MS-50-RF equipped with FAB source. The  $[\text{M}+\text{H}]^+$  ions were peakmatched using ions derived from the glycerol matrix. Thin layer chromatography (TLC) analyses were carried out with use of TLC plates 60 F<sub>254</sub> purchased from Merck and were visual-



ized in an UV light (254 and/or 343 nm) and/or with a ninhydrin spray reagent (0.3 g ninhydrin in 100 cm<sup>3</sup> butan-1-ol and 3 cm<sup>3</sup> HOAc) for azasugars and its derivatives. The silica gel (0.063-0.200) used for column chromatography was purchased from Merck. ODNs were synthesised on an Assembler Gene Special DNA-Synthesizer (Pharmacia Biotech). Purification of 5'-O-DMT-on and 5'-O-DMT-off ODNs were accomplished using a Waters Delta Prep 4000 Preparative Chromatography System. The modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. All solvents were distilled before use. The reagents used were purchased from Aldrich, Sigma or Fluka. The reagents for Gene Assembler were purchased from Cruachem (UK).

***N*-(Pyren-1-ylmethyl)-(3*R*,4*S*)-4-[(1*S*)-1,2-dihydroxyethyl]pyrrolidin-3-ol (3)**

**Method A.** Azasugar **1** (50 mg, 0.34 mmol) was dissolved in DMF (5 cm<sup>3</sup>), 1-(chloromethyl)pyrene (103 mg, 0.41 mmol) and Et<sub>3</sub>N (0.057 cm<sup>3</sup>, 0.41 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×5 cm<sup>3</sup>). The residue was chromatographed on a silica gel column with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (0-20%, v/v) as eluent affording the pure product **3** (70 mg, 57%): *R*<sub>f</sub> 0.20 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); δ<sub>H</sub>(CD<sub>3</sub>OD) 2.36 (1 H, m, H-4), 2.95 (1 H, m, H-5), 3.08 (1 H, dd, *J* 2.8 and 10.5, H-2), 3.22 (1 H, dd, *J* 5.4 and 12.0, H-5), 3.34 (1 H, m, H-2), 3.50-3.65 (3 H, m, CH[OH]CH<sub>2</sub>OH), 4.42 (1 H, m, H-3), 4.65 (2 H, s, CH<sub>2</sub>pyren-1-yl), 4.88 (3 H, br. s, 3×OH), 7.90-8.40 (9 H, m, H<sub>arom</sub>); δ<sub>C</sub>(CD<sub>3</sub>OD) 50.7 (C-4), 56.7 (C-5), 57.2 (C-2), 62.7 (CH<sub>2</sub>pyren-1-yl), 65.7 (CH<sub>2</sub>OH), 71.8 (CH[OH]), 72.7 (C-3), 123.8, 125.5, 125.8, 125.9, 126.6, 126.8, 127.3, 128.0, 128.2, 129.1, 129.4, 129.9, 131.2, 131.9, 132.5, 133.2 (pyren-1-yl); *m/z* (FAB) 362.1748 [M+H]<sup>+</sup>, C<sub>23</sub>H<sub>24</sub>NO<sub>3</sub> requires 362.1756.

**Method B.** Azasugar **1** (70 mg, 0.48 mmol) was dissolved in DMF/EtOH (3:1, 10 cm<sup>3</sup>) and 1-pyrenecarbaldehyde (270 mg, 1.18 mmol) and NaCNBH<sub>3</sub> (74 mg, 1.18 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. Concentrated HCl was added until pH<2. Solvent was evaporated under reduced pressure, co-evaporated with toluene (2×5 cm<sup>3</sup>). The residue was purified using silica gel column chromatography with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (0-20%, v/v) affording the compound **3** (110 mg, 63%).

***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-4-(hydroxymethyl)pyrrolidin-3-ol (4)**

**Method A.** Azasugar **2** (100 mg, 0.86 mmol) was dissolved in DMF (10 cm<sup>3</sup>) and 1-(chloromethyl)pyrene (257 mg, 1.03 mmol) and Et<sub>3</sub>N (0.140 cm<sup>3</sup>, 1.03 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×5 cm<sup>3</sup>). The residue was dissolved in H<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub> (1:1, 40 cm<sup>3</sup>) and the water layer was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic fractions were dried (Na<sub>2</sub>SO<sub>4</sub>), evaporated *in vacuo* and chromatographed on a silica gel column with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (0-20%, v/v) affording the title compound **4** (130 mg, 46%); *R*<sub>f</sub> 0.17 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); δ<sub>H</sub>(CDCl<sub>3</sub>) 2.08 (1 H, m, H-4), 2.29 (1 H, m, H-5), 2.57 (1 H, dd, *J* 2.8 and 10.2, H-2), 2.85 (2 H, m, H-2 and H-5), 3.43 (2 H, s, CH<sub>2</sub>OH), 3.47 (2 H, br. s, 2×OH), 4.08 (1 H, m, H-3), 4.19 (2 H, s, CH<sub>2</sub>pyren-1-yl), 7.90-8.40 (9 H, m, H<sub>arom</sub>); δ<sub>C</sub>(CDCl<sub>3</sub>) 49.8 (C-4), 55.9 (C-5), 57.5 (C-2), 62.3 (CH<sub>2</sub>pyren-1-yl), 64.2 (CH<sub>2</sub>OH), 73.9 (C-3), 123.3, 124.4, 124.6, 124.8, 125.1, 125.9, 127.3, 127.6, 127.8, 129.5, 130.7, 130.9, 131.1 (pyren-1-yl); *m/z* (FAB) 332.1631 [M+H]<sup>+</sup>, C<sub>23</sub>H<sub>24</sub>NO<sub>3</sub> requires 332.1651.

**Method B.** Azasugar **2** (1.18 g, 10.1 mmol) was dissolved in DMF/EtOH (3:1, 150 cm<sup>3</sup>) and 1-pyrene-carbaldehyde (3.47 g, 15.1 mmol) and NaCNBH<sub>3</sub> (950 mg, 15.1 mmol) were added. The reaction mixture was stirred at room temp. under nitrogen overnight. Concentrated HCl was added until pH<2. The solvent was evaporated under reduced pressure and co-evaporated with toluene (2×50 cm<sup>3</sup>). The residue was dissolved in H<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub> (1:1, v/v, 150 cm<sup>3</sup>) and the water layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3×75 cm<sup>3</sup>). The combined organic fractions were dried (Na<sub>2</sub>SO<sub>4</sub>), evaporated under diminished pressure. The residue was purified using silica gel column chromatography with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (0-20%, v/v) affording the compound **4** as an oil which crystallised on standing (1.9 g, 57%), mp 104 - 105 °C.

**Method C.** A cooled solution of compound **3** (110 mg, 0.304 mmol) in EtOH (4 cm<sup>3</sup>) was added to a solution of NaIO<sub>4</sub> (71.6 mg, 0.335 mmol) in H<sub>2</sub>O (1.5 cm<sup>3</sup>) under stirring. After 30 min NaBH<sub>4</sub> (12.3 mg, 0.335 mmol) was added. After 30 min the resulting solution was acidified with 2M HCl until pH 2 under vigorous stirring. The solvent was removed *in vacuo*. The residue was dissolved in H<sub>2</sub>O/CH<sub>2</sub>Cl<sub>2</sub> (1:1, v/v, 20 cm<sup>3</sup>) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (4×15 cm<sup>3</sup>). The combined organic layers were

dried ( $\text{Na}_2\text{SO}_4$ ), evaporated under diminished pressure to dryness affording compound **4** (40 mg, 40%).

***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-4-[(4,4'-dimethoxytriphenylmethoxy)methyl]pyrrolidin-3-ol (**5**)**

Compound **4** (139 mg, 0.42 mmol) was dissolved in anhydrous pyridine (10  $\text{cm}^3$ ) and DMTCl (178 mg, 0.53 mmol) was added. The mixture was stirred for 24 h under nitrogen at room temp. MeOH (1  $\text{cm}^3$ ) was added to quench the reaction and the solvents were evaporated under reduced pressure and co-evaporated with toluene (2x5  $\text{cm}^3$ ). The residue was re-dissolved in  $\text{H}_2\text{O}/\text{CH}_2\text{Cl}_2$  (1:1, v/v, 20  $\text{cm}^3$ ), and the mixture was washed with saturated aqueous  $\text{NaHCO}_3$ . The organic layer was dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated under reduced pressure. Purification using silica gel column chromatography (5-40% EtOAc/cyclohexane, v/v) gave the title compound **5** as a foam (160 mg, 61%) which was used in the next step without further purification:  $R_f$  0.45 (49% EtOAc/49% cyclohexane/2%  $\text{Et}_3\text{N}$ , v/v/v);  $\delta_{\text{H}}$ ( $\text{CDCl}_3$ ) 2.20 (1 H, m, H-4), 2.34 (1 H, m, H-5), 2.53 (1 H, br.s, OH), 2.62 (1 H, dd,  $J$  5.6 and 9.9, H-2), 2.72 (1 H, dd,  $J$  2.5 and 9.8, H-2), 3.06 (3 H, m,  $\text{CH}_2\text{ODMT}$  and H-5), 3.71 (6 H, s,  $\text{OCH}_3$ ), 4.01 (1 H, m, H-3), 4.21 (2 H, s,  $\text{CH}_2\text{pyren-1-yl}$ ), 6.78 (4 H, m, DMT), 7.10-7.40 (9 H, m, DMT), 7.90-8.40 (9 H, m,  $\text{H}_{\text{arom}}$ );  $\delta_{\text{C}}$ ( $\text{CDCl}_3$ ) 48.8 (C-4), 55.2 ( $\text{OCH}_3$ ), 56.1 (C-5), 58.0 (C-2), 61.9 ( $\text{CH}_2\text{pyren-1-yl}$ ), 64.5 ( $\text{CH}_2\text{OH}$ ), 74.9 (C-3), 85.9 (C- $\text{Ar}_3$ ), 113.0, 123.8-132.3 (DMT and pyren-1-yl), 144.9, 158.4 (DMT);  $m/z$  (FAB) 634.2740  $[\text{M}+\text{H}]^+$ ,  $\text{C}_{44}\text{H}_{42}\text{NO}_5$  requires 634.2722.

***N*-(Pyren-1-ylmethyl)-(3*R*,4*R*)-3-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-4-[(4,4'-dimethoxytriphenylmethoxy)methyl]pyrrolidine (**6**)**

Compound **5** (140 mg, 0.22 mmol) was dissolved under nitrogen in anhydrous  $\text{CH}_2\text{Cl}_2$  (5  $\text{cm}^3$ ). *N,N*-Diisopropylammonium tetrazolide (61 mg, 0.42 mmol) was added followed by dropwise addition of 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphane (0.140  $\text{cm}^3$ , 0.44 mmol). After 2.0 h analytical TLC showed no more starting material and the reaction was quenched with  $\text{H}_2\text{O}$  (1  $\text{cm}^3$ ) followed by addition of  $\text{CH}_2\text{Cl}_2$  (10  $\text{cm}^3$ ). The mixture was washed with saturated aqueous  $\text{NaHCO}_3$  (2x10  $\text{cm}^3$ ). The organic phase was dried ( $\text{Na}_2\text{SO}_4$ ) and the solvents were removed under reduced pressure. The residue was purified using silica gel column chromatography with cyclohexane/EtOAc (0-20%, v/v). Combined UV-active fractions were evapo-

rated *in vacuo* affording **6** (158 mg, 57%) as foam that was co-evaporated with dry acetonitrile ( $3 \times 30 \text{ cm}^3$ ) before using it in ODN synthesis.  $R_f$  0.85 (49% EtOAc/49% cyclohexane/2% Et<sub>3</sub>N, v/v/v);  $\delta_H$ (CDCl<sub>3</sub>) 0.93 (6 H, m, CH<sub>3</sub> [Pr<sup>i</sup>]), 1.04 (6 H, m, CH<sub>3</sub> [Pr<sup>i</sup>]), 2.30 (2 H, m, H-4 and H-5), 2.48 (2 H, m, CH<sub>2</sub>CN), 2.64 (1 H, m, H-2), 2.78 (1 H, m, H-2), 2.98 (2 H, m, OCH<sub>2</sub>CH<sub>2</sub>CN), 3.08 (1 H, m, H-5), 3.50 (4 H, m, CH [Pr<sup>i</sup>] and CH<sub>2</sub>ODMT), 3.65 (6 H, s, OCH<sub>3</sub>), 4.01 (1 H, m, H-3), 4.20 (2 H, m, CH<sub>2</sub>pyren-1-yl), 6.68 (4 H, m, DMT), 7.05-7.40 (9 H, m, DMT), 7.85-8.40 (9 H, m, H<sub>arom</sub>);  $\delta_P$ (CDCl<sub>3</sub>) 148.2 (s), 149.0 (s) in the ratio 2:1.

#### Synthesis and purification of modified and unmodified oligodeoxynucleotides

The oligodeoxynucleotides were synthesised on a Pharmacia Gene Assembler<sup>®</sup> Special synthesizer in 0.2  $\mu\text{mol}$ -scale (7.5  $\mu\text{mol}$  embedded per cycle, Pharmacia primer support<sup>TM</sup>) using commercially available 2-cyanoethylphosphoramidites and **6**. The synthesis followed the regular protocol for the DNA synthesizer. The coupling time for **6** was increased from 2 to 24 min and the cycle was repeated twice. The 5'-O-DMT-on ODNs were removed from the solid support and deprotected with 32% aqueous NH<sub>3</sub> (1  $\text{cm}^3$ ) at 55 °C for 24 h and then purified on preparative HPLC using a Hamilton PRP-1 column. The solvent systems were buffer A [950  $\text{cm}^3$  0.1 M NH<sub>4</sub>HCO<sub>3</sub> and 50  $\text{cm}^3$  CH<sub>3</sub>CN (pH = 9.0)] and buffer B [250  $\text{cm}^3$  0.1 M NH<sub>4</sub>HCO<sub>3</sub> and 750  $\text{cm}^3$  CH<sub>3</sub>CN (pH = 9.0)] which were used in the following order: 5 min A, 30 min liner gradient of 0-70% B in A, 5 min liner gradient of 70-100% B in A. Flow rate was 1  $\text{cm}^3 \text{ min}^{-1}$ . The purified 5'-O-DMT-on ODNs eluted as one peak after approximately 30 min [UV control 254 nm and 343 nm (for pyrene containing ODNs)]. The fractions were concentrated *in vacuo* followed by treatment with 10% aqueous HOAc (1  $\text{cm}^3$ ) for 20 min and further purification on HPLC under the same conditions to afford detritylated ODNs which eluted at 23-28 min. The purity of oligos synthesised was 99-100% according to the preparative HPLC. The resulted solutions were evaporated *in vacuo* and co-evaporated twice with water to remove volatile salts to afford ODNs, which were used in melting temperature measurements. All oligonucleotides containing pyrenylmethylazasugar derivative **6** were confirmed by MALDI-TOF analysis (entry **C**: found 4005.65, calcd. 4005.76; entry **D**: 4398.02, calcd. 4398.87; entry **F3**: found 4903.05, calcd. 4904.89).

#### Melting experiments

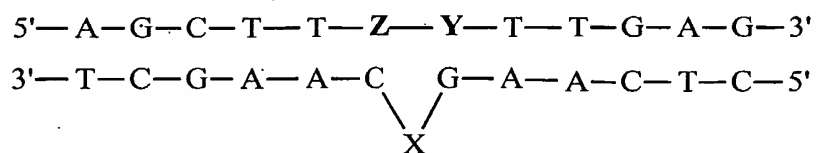
Melting temperature measurements were performed on a Perkin-Elmer UV/VIS spectrometer fitted with a PTP-6 Peltier temperature-programming element. The absorbance at 260 nm was measured from 18 °C to 85 °C in 1 cm cells. The melting temperature was determined as the maximum of the derivative plots of the melting curve. The oligodeoxynucleotides were dissolved in a medium salt buffer (pH = 7.0, 1 mM EDTA, 10 mM Na<sub>2</sub>HPO<sub>4</sub>×2H<sub>2</sub>O, 140 mM NaCl) to a concentration of 1.0 μM for each strand.

### Example 27

### Fluorescence when hybridized to mismatched targets

Quenching in fluorescence is a sign of strong interaction of the fluorophore with the duplex. Structural minimization calculations have supported that the pyrene moiety is intercalated into the duplex. It was therefore anticipated that the introduction of mismatches near the site of intercalation results in increased flexibility to the pyrene and hence increased fluorescence. This was also what was found irrespective to which side of the intercalator a mismatch is introduced (Table 6).

**Table 6: Fluorescent data of mono modified ODN hybridised to either the complementary sequence or to one of six different neighbouring single point mutants.**

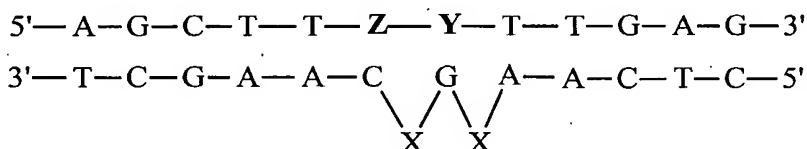


Name	Z	Y	382 nm Rel. In- tensity	395 nm Rel. In- tensity	480 nm. Rel. In- tensity
Probe alone	--	--	48	40	1
Wt	G	C	15	12	1
Mut. 1	C	C	59	50	2
Mut. 2	A	C	75	63	2

Mut. 3	T	C	50	42	2
Mut. 4	G	T	34	29	2
Mut. 6	G	A	63	53	2

To test the hypothesis that probe III could be used for detection of single point mutants, it was hybridised to target with all four variants of Y, and the intensity of the excimer band at 480 nm was significantly increased (Table 7) when a mismatch was introduced (Y = G, A, T). Again it was expected that the fluorescence of the bands at 382 and 395 nm would increase upon introduction of a mismatch, which was also observed. Surprisingly the fluorescence at 480 nm also increased with introduction of a mismatch at the 3' side of both intercalators (Z = C, A, T), indicating that the two pyrene moieties are able to interact with each other (Table 7). This would only be expected if a loop, large enough to let the pyrene moieties interact, is created. It is noteworthy that the fluorescence at 382 and 395 is quenched when hybridised to a complementary sequence, but increased when hybridizing to a sequence with one mismatch. It should therefore be possible to use all three wavelengths (382, 295 and 480) to differentiate between a fully complementary sequence and a complementary sequence with one mismatch.

**Table 7: Fluorescent data of a double modified ODN hybridised to either the complementary sequence or to one of six different single point mutants.**



Name	Z	Y	382 nm Rel. In- tensity	395 nm Rel. In- tensity	480 nm Rel. In- tensity
SsDNA	--	--	44	38	17
Wt	G	C	19	17	4
Mut. 1	C	C	84	73	14
Mut. 2	A	C	74	64	10

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Mut. 3	T	C	84	74	12.5
Mut. 4	G	T	62	54	8
Mut. 5	G	G	84	74	17
Mut. 6	G	A	70	60	12

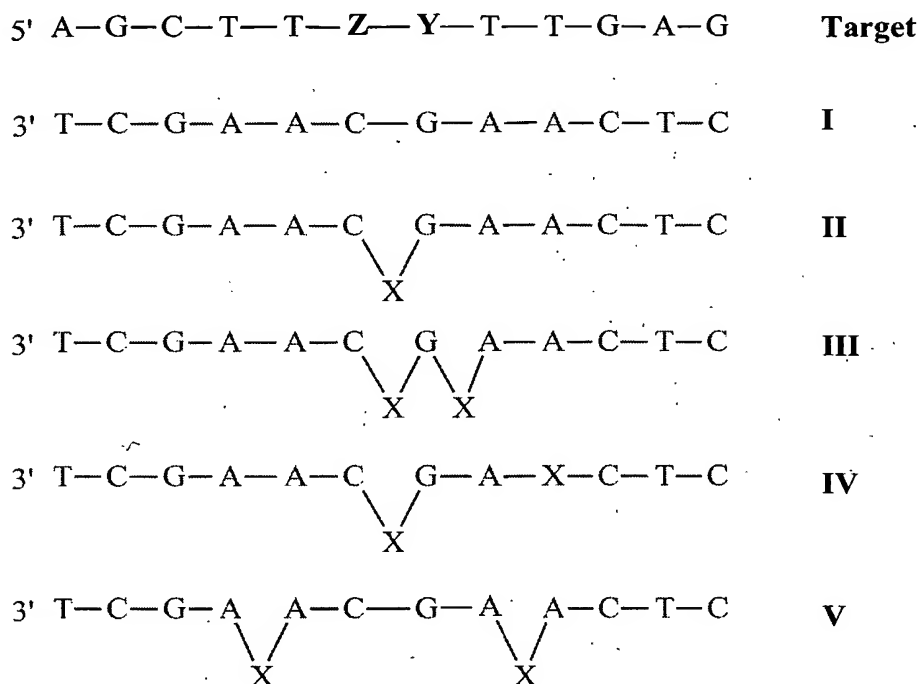
### Example 28

#### **Thermal denaturation studies.**

Insertions of intercalators into DNA have been accompanied by decrease in specificity for hybridization to fully complementary sequences when compared to sequences with mismatches in the basepairs surrounding the intercalator. Experiments were aimed to test if this is also the case for duplexes with pyrene moiety insertions, placing mismatches to either side of the intercalator, next to and between two intercalators

The specificity is measured by the difference in the melting temperature between the fully complementary duplex and the duplex where one mismatch has been introduced. Melting temperature data are presented in Table 8.

**Table 8: Melting temperature data for ODNs with different insertion patterns hybridised to either the complementary strand or one of the three possible point mutations at nucleotide #6 and #7. X=1.**



Tar- get		Probe										
		Z	Y	I	II		III		IV		V	
				[°C]	[°C]		[°C]		[°C]		[°C]	
Wt	G C	47.4	ΔT	50.4	ΔT	51.4	ΔT	45.4	ΔT	60.8	ΔT	
Mut. 1	C C	23.4	-24.0	34.0	-16.0	38.0	-13.4	23.4	-22.0	33.8	-27.0	
Mut. 2	A C	30.8	-16.6	34.2	-16.2	36.6	-14.8					
Mut. 3	T C	27.6	-18.8	33.6	-16.8	35.2	-16.2	25.4	-20.0	37.4	-23.4	
Mut. 4	G T	36.2	-11.2	42.2	-8.2	45.2	-6.2	36.6	-8.8	45.8	-15.0	
Mut. 5	G G	40.0	-7.4	42.4	-8.0	38.6	-12.8	39.4	-6.0	53.2	-7.6	
Mut. 6	G A	39.8	-7.6	39.0	-11.4	39.0	-12.4	39.2	-6.2	49.0	-11.8	



As seen from Table 8 the specificity against mismatches of the modified probe is in the range of that of the unmodified probe, though there seems to be a drop in selectivity for C-C mismatches to the 5' end of the intercalation site (Table 8; Mut. 1 with Probe II and III). The only other consistent trend is that the double modified probe where the two intercalators are separated by four nucleotides (probe V) is more specific against mismatches two nucleotides away from any of the intercalation sites than the unmodified probe (probe I). The rest of the melting temperature differences are close to the values for the unmodified duplexes. Most important in respect to the search for single point mutants is that probe III is selective for its target having the right base in between the pyrene moieties, which is the case and in two out of the three possible mismatches it is even more specific, being less specific in the last instance (Table 8).

#### Example 29

##### Beacon-primers

An example of a Beacon-design primer is given in figure 40. The primer consists of 39 nucleotides, which are designed so that they can form a stem-loop structure.

The primer has a target-complementary region, that is complementary to the target DNA, which is 24 nucleotides long. Furthermore, the primer has a self-complementary region, that is capable of hybridising to the other end of the primer. The self-complementary region is 15 nucleotides long and comprises furthermore 4 intercalator pseudonucleotides. Two of the intercalator pseudonucleotides are positioned so they are capable of forming an intramolecular excimer.

The melting temperature of the primer/target hybrid is around 67°C, whereas the melting temperature of the selfhybrid is around 46°C.

The beacon primer can be used for PCR and allows quantification of the PCR.

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Target specific beacon primers and template DNA is provided (figure 41A). The beacon primers and the template DNA is stored at 0-4°C.

Double stranded DNA is denatured at 94°C (figure 41B). The beacon primers are annealed to the target DNA at around 65°C (figure 41C) and the primers are elongated by Taq polymerase.

Subsequently, the temperature is lowered to around 45°C and unhybridised beacon primers are hybridised to itself (figure 41D). Excimer fluorescence is determined and correlated to the amount of elongated beacon primer.

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**Claims**

- 5 1. A hairpin oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide of the general structure



10 wherein

X is a backbone monomer unit capable of being incorporated into the phosphate backbone of a nucleic acid; and

15 Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid ; and

Y is a linker moiety linking said backbone monomer unit and said intercalator; and

20 wherein the oligonucleotide comprises a first sequence consisting of n nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides and a second nucleotide sequence consisting of m nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides, wherein said first sequence is capable of hybridising to said second sequence.

25

2. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the first sequence and the second sequence are separated by a third sequence consisting of p nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides.

30

3. The oligonucleotide or oligonucleotide analogue according to claim 2, wherein the second and third sequence are capable of hybridising to a corresponding target sequence comprised within a nucleic acid or nucleic acid analogue and

wherein the second and third sequence are capable of priming a template directed extension reaction.

4. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
5 is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.
5. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
10 is an integer in the range from 5 to 10, such as from 10 to 15, for example from 15 to 20, such as from 20 to 30, for example from 30 to 50.
6. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
is equal to n.
- 15 7. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
the oligonucleotide comprises between 2 and 5, such as between 5 and 10,  
such as between 10 and 15, for example between 15 and 20 intercalator  
pseudonucleotides.
- 20 8. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
the first sequence comprises at least one, for example between 2 and 5, such as  
between 5 and 10, such as between 10 and 15, for example between 15 and 20  
intercalator pseudonucleotides.
- 25 9. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
the second sequence comprises no intercalator pseudonucleotide.
10. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
30 the first sequence comprises at least 2 intercalator pseudonucleotides, wherein  
the intercalators of said intercalator pseudonucleotides are capable of forming  
an intramolecular excimer, an intramolecular exciplex, FRET or a charge  
transfer complex, when the first sequence is not hybridised with the second  
sequence or another nucleic acid.

11. The oligonucleotide or oligonucleotide analogue according to claim 2, wherein p is an integer between 5 and 10, such as between 10 and 15, for example between 15 and 20, such as between 20 and 30, for example between 30 and 50.

5

12. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the oligonucleotide consists of in the range from 15 to 50 nucleotides.

10

13. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit (X) is selected from the group consisting of acyclic backbone monomer units.

15

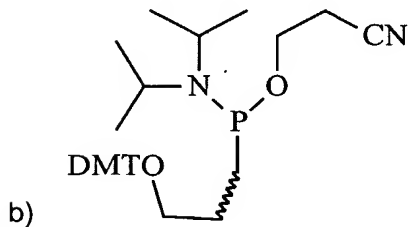
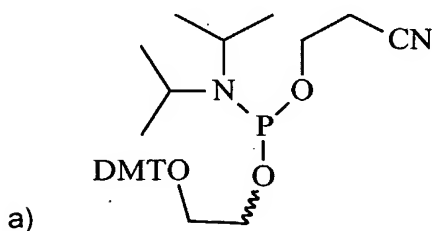
14. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit is selected from the group consisting of acyclic backbone monomer units capable of stabilising a bulge insertion.

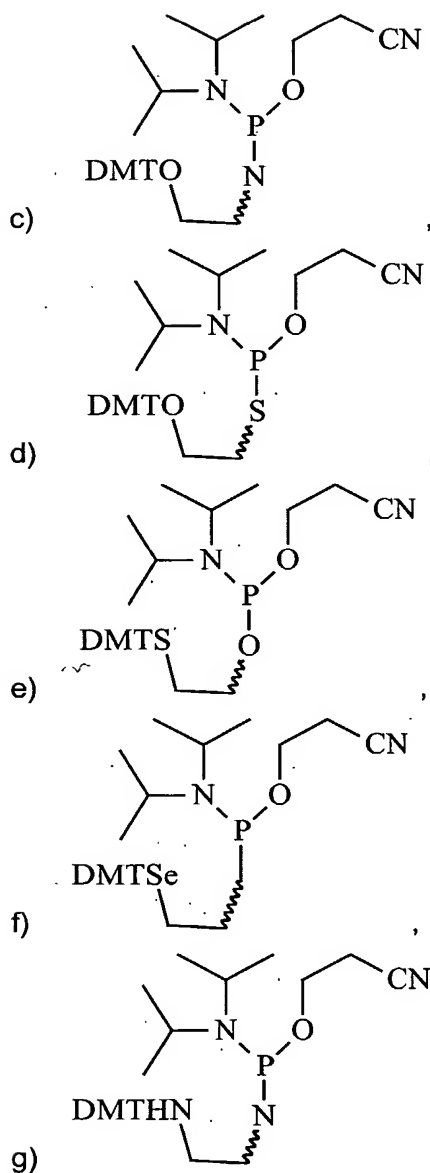
20

15. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one backbone monomer unit is selected from the group consisting of phosphoramidites.

16. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the backbone monomer units are selected from the group consisting of backbone monomer units with the following structures

25





17. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
the intercalators comprise a chemical group selected from the group consisting  
of polyaromates and heteropolyaromates.
18. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein  
the intercalators are selected from the group consisting of polyaromates and  
heteropolyaromates.



19. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalators are selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.
20. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one intercalator is pyrene.
21. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the linkers comprise a chain of x atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb.
22. The oligonucleotide or oligonucleotide analogue according to claim 21, wherein x is selected from the group of integers from 1 to 7, preferably from 2 to 5, most preferably 3.
23. The oligonucleotide or oligonucleotide analogue according to claim 21, wherein the chain is substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.
24. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is an alkyl chain.
25. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is alkyl chain substituted with one or more selected from the group consisting C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.
26. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein at least one linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb.
27. The oligonucleotide or oligonucleotide analogue according to claim 26, wherein the linker is substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

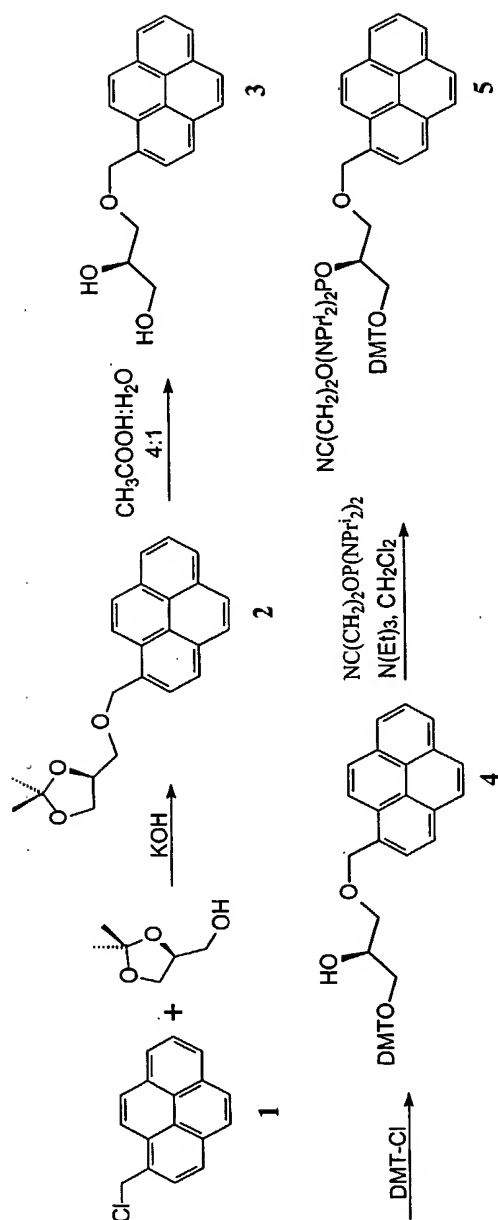
- 5 28. The oligonucleotide or oligonucleotide analogue according to claim 1, wherein the intercalator pseudonucleotide is the phosphoramidite of 1-(4,4'-dimethoxytriphenylmethoxy)-3-pyrenemethoxy-2-propanol.
- 10 29. A method of detecting hybridisation between a hairpin oligonucleotide or oligonucleotide analogue according to any of claims 1 to 28 and a template comprising a nucleic acid sequence capable of hybridising to at least the second sequence comprising the steps of
- 15 a) providing the template; and  
b) providing an oligonucleotide or oligonucleotide analogue according to any of claims 1 to 28; and  
c) incubating the template and the oligonucleotide or oligonucleotide analogue under conditions allowing for hybridisation; and  
d) detecting hybridisation.
- 20 30. The method according to claim 29, wherein step d) comprises determining spectral properties of the oligonucleotide or oligonucleotide analogue.
31. The method according to claim 30, wherein a spectral signal above a predetermined limit is indicative of hybridisation.
- 25 32. The method according to claim 30, wherein the spectral properties are fluorescent properties.
- 30 33. The method according to claim 30, wherein the spectral properties are monomer fluorescence and/or excimer fluorescence and/or exciplex fluorescence and/or FRET and/or charge transfer fluorescence.
34. The method according to claim 29, wherein step d) comprises determining melting temperature.
- 35 35. The method according to claim 34, wherein a melting temperature above a predetermined limit is indicative of hybridisation.

36. The method according to claim 29, wherein the template comprises a nucleic acid sequence capable of hybridising with the second sequence and the third sequence.
- 5
37. The method according to claim 29, wherein the template does not comprises a nucleic acid sequence capable of hybridising with the first sequence.
38. A method for detecting template directed extension of an oligonucleotide analogue comprising at least one intercalator pseudonucleotides, said method comprising the steps of
- 10
- a) providing the template(s); and
  - b) providing at least one oligonucleotide analogue comprising intercalator pseudonucleotide(s); and
  - 15 c) incubating said template(s) and said oligonucleotide analogue under conditions allowing for hybridization; and
  - d) providing nucleotides and/or nucleotide analogues and/or intercalator pseudonucleotides; and
  - 20 e) extending the oligonucleotide analogue in a templated directed manner, thereby obtaining an extension product; and
  - f) detecting said template directed extension.
39. The method according to claim 38, wherein the oligonucleotide analogue comprises a first sequence and a second sequence and wherein the second sequence is capable of hybridising with the template.
- 25
40. The method according to claim 38, wherein the oligonucleotide analogue is an oligonucleotide analogue according to any of claims 1 to 28.
- 30
41. The method according to claim 40, wherein the second sequence is capable of hybridising to the template.
42. The method according to claim 38, wherein step f) comprises determining spectral properties of the extension product.
- 35

43. The method according to claim 42, wherein a spectral signal above a predetermined limit is indicative of the presence of an extension product.
- 5      44. The method according to claim 42, wherein the spectral properties are fluorescent properties.
45. The method according to claim 42, wherein the spectral properties are monomer fluorescence and/or excimer fluorescence and/or exciplex fluorescence and/or
- 10      FRET and/or charge transfer fluorescence.
46. The method according to claim 38, wherein step f) comprises determining melting temperature.
- 15      47. The method according to claim 46, wherein a melting temperature above a predetermined limit is of the presence of an extension product.
48. The method according to claim 38, wherein step e) involves the use of an enzyme selected from the group consisting of ligase and DNA polymerases.
- 20      49. The method according to claim 38, wherein the method is used in a polymerase chain reaction (PCR).
- 25

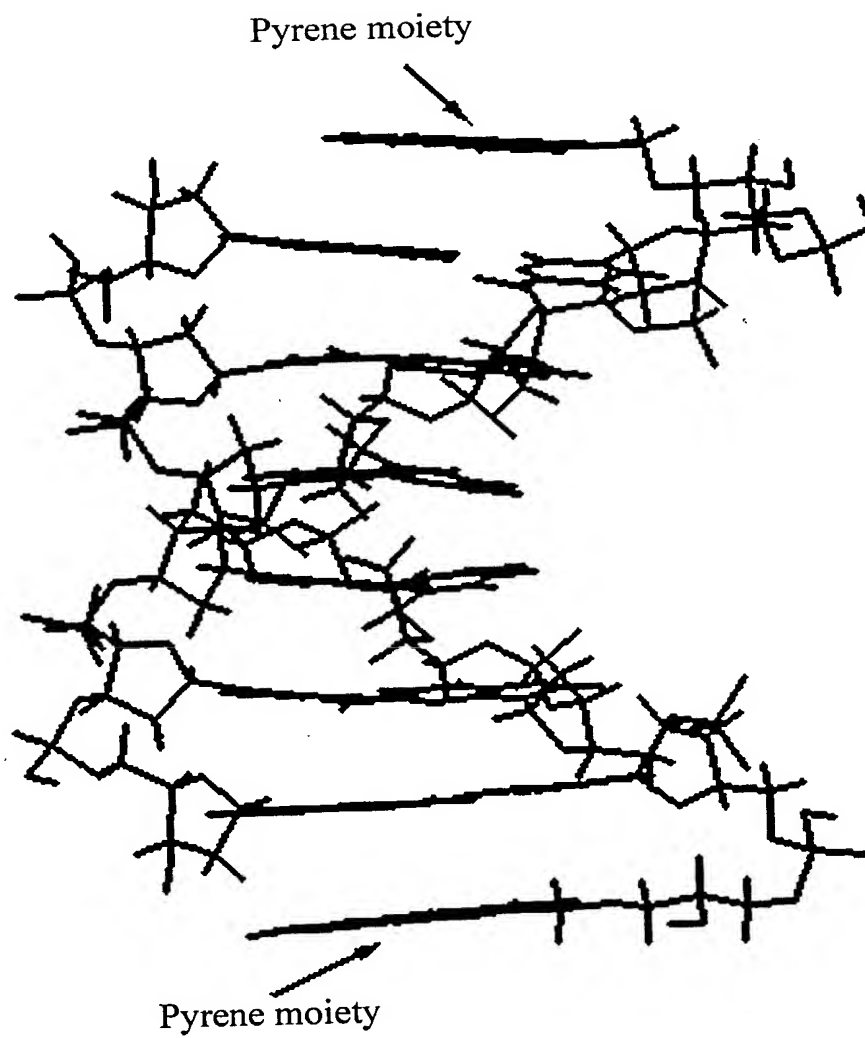
1/32

Fig. 1



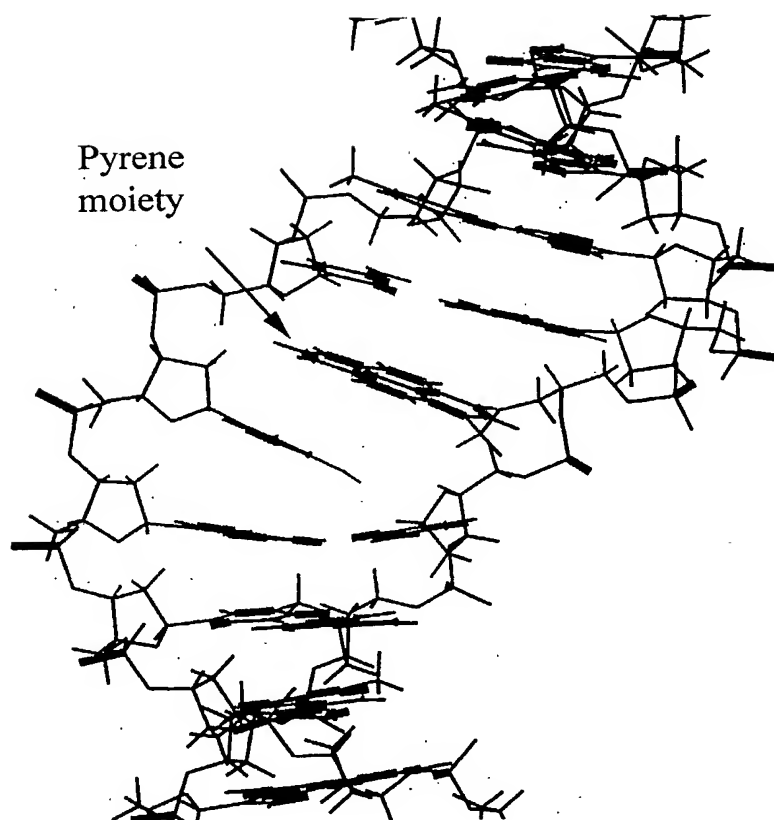
2/32

Fig. 2



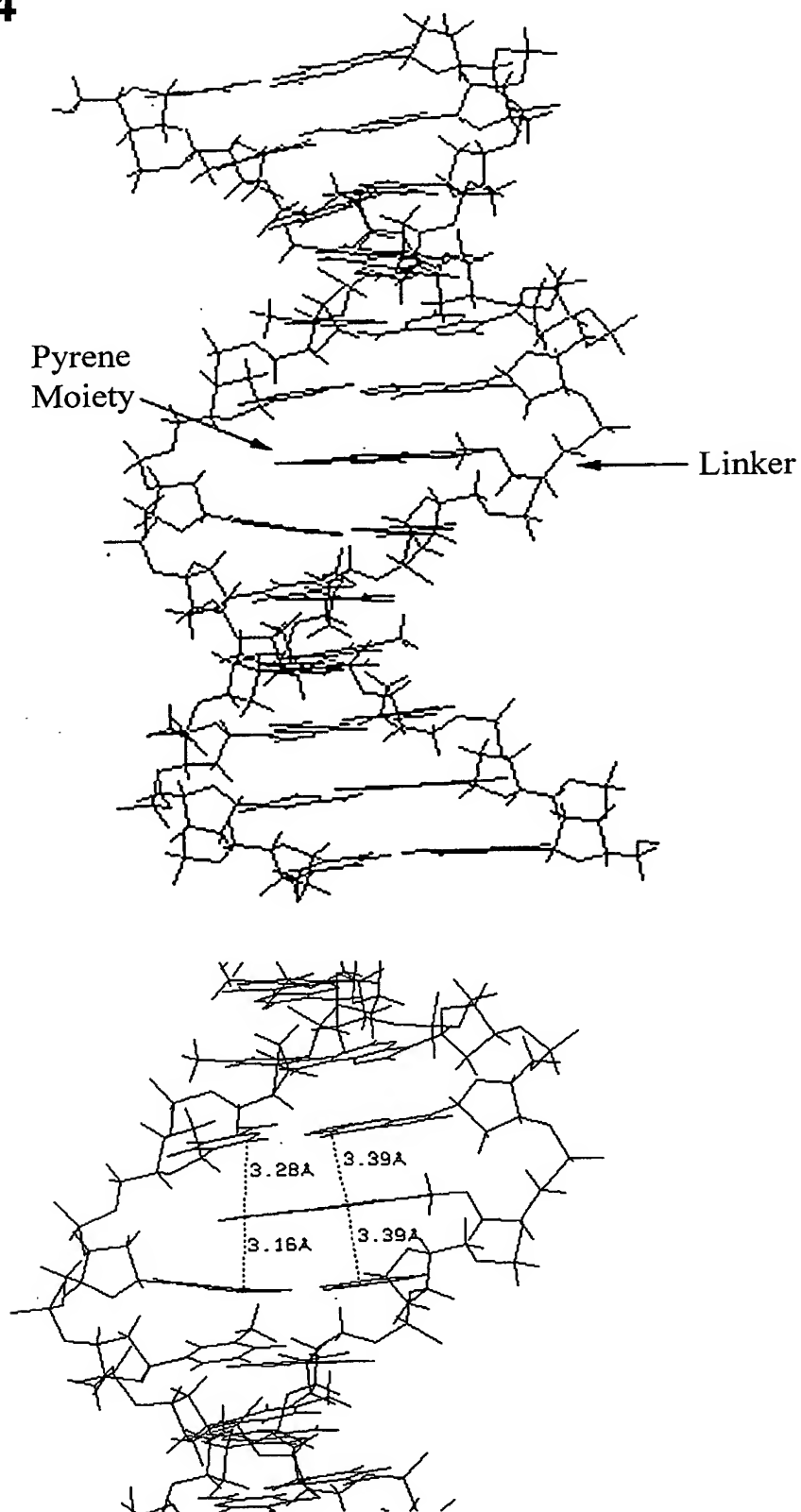
3/32

Fig. 3



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Fig. 4





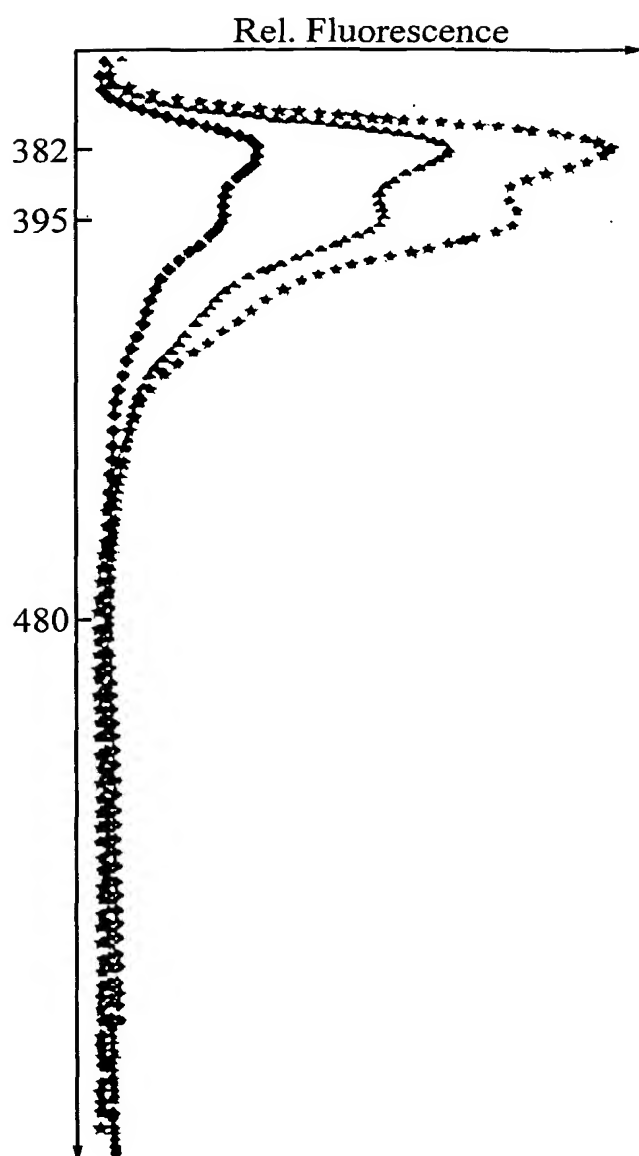
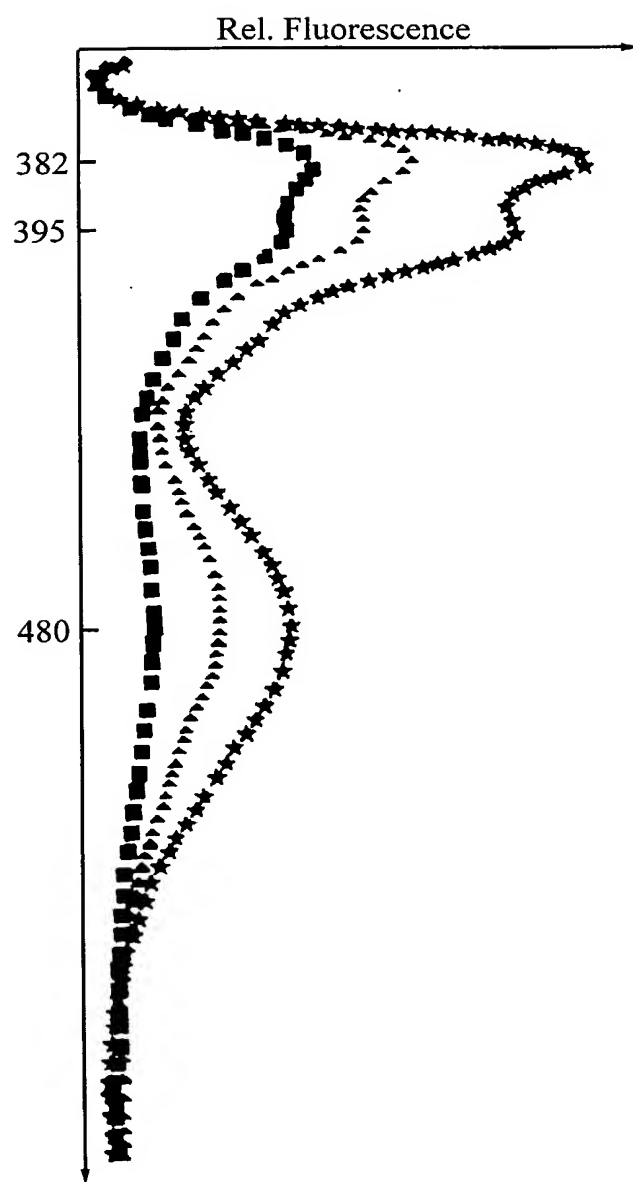
**5/32****Fig. 5**

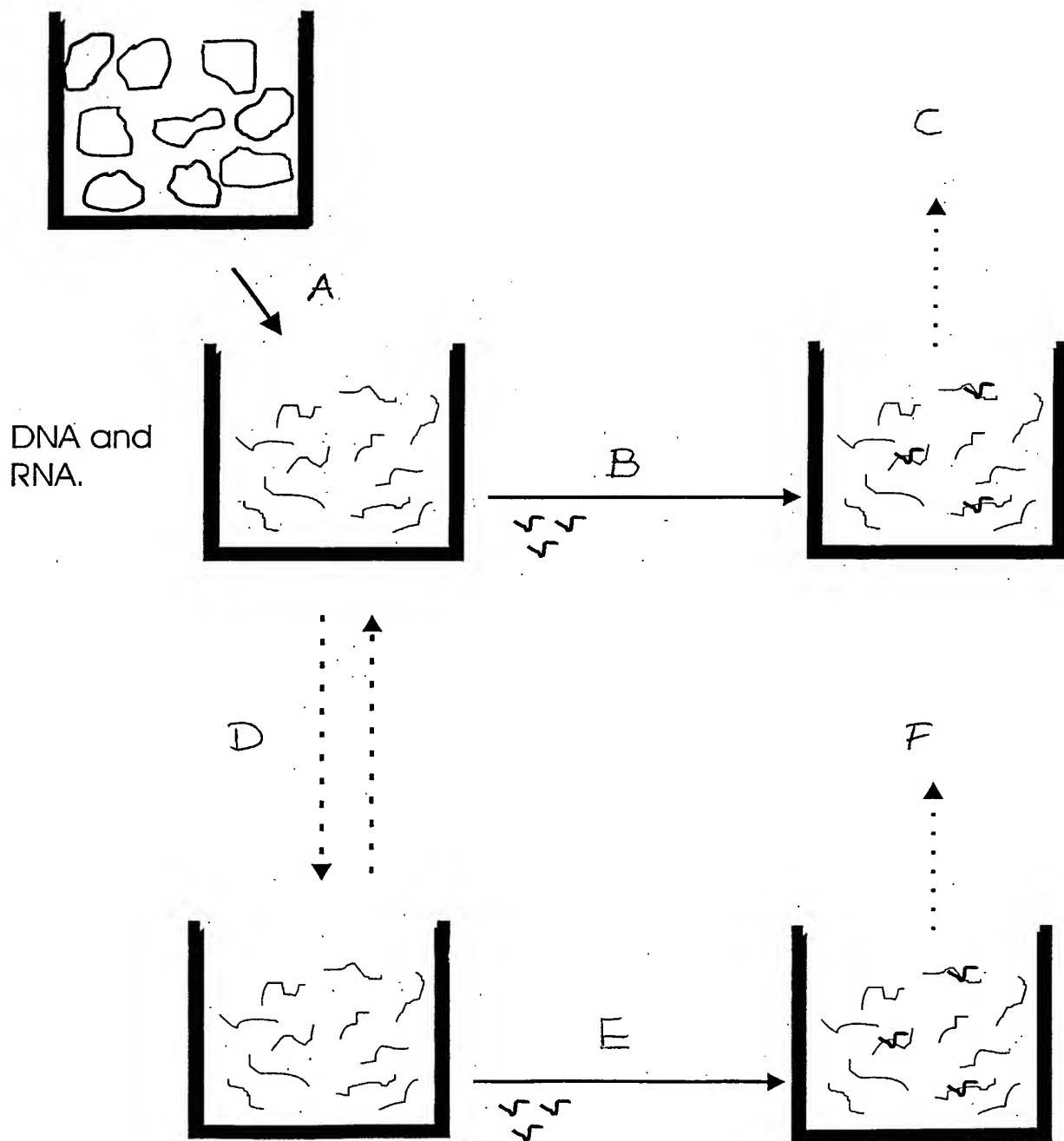
Fig. 6

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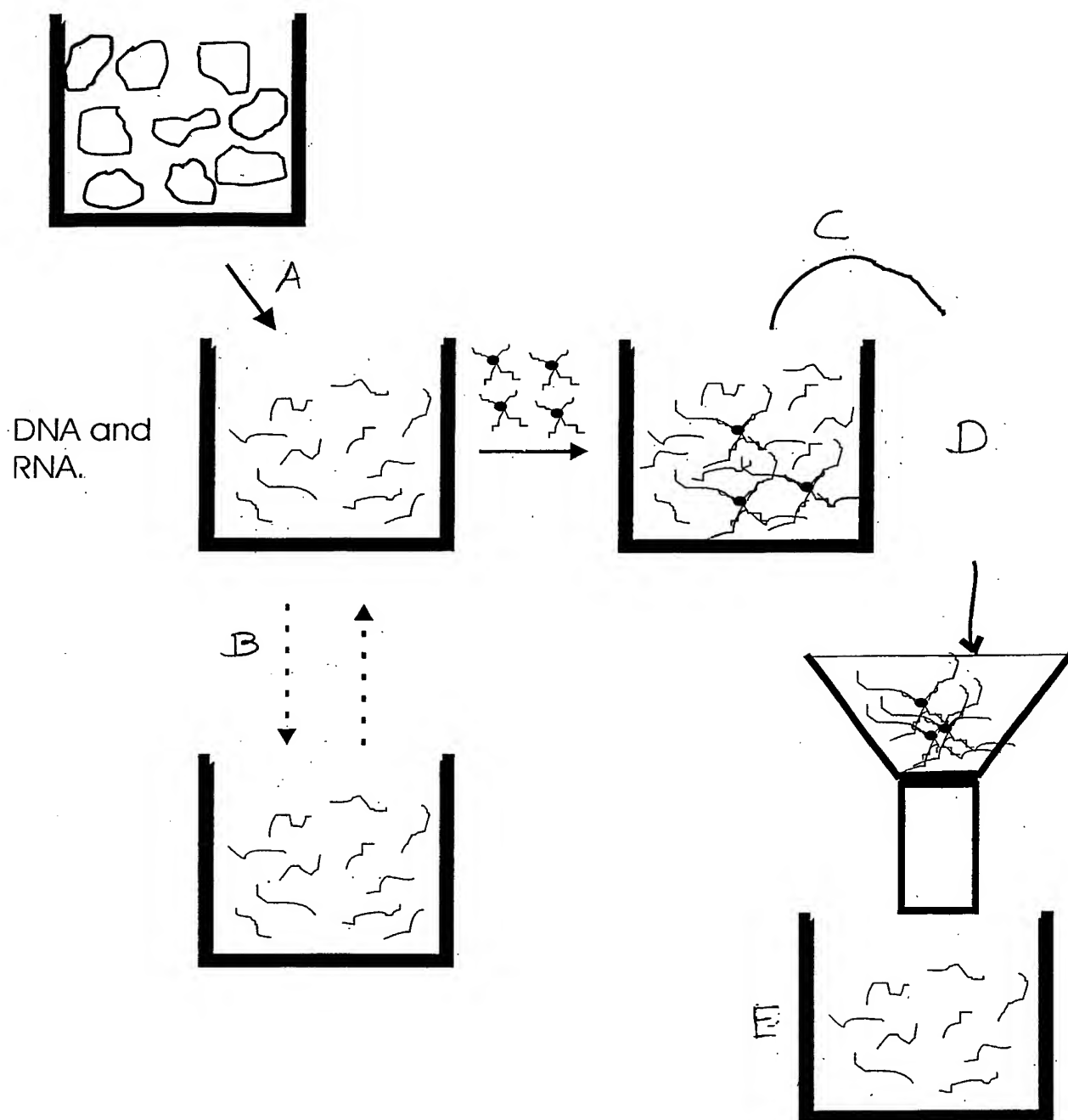
7/32

Fig. 7



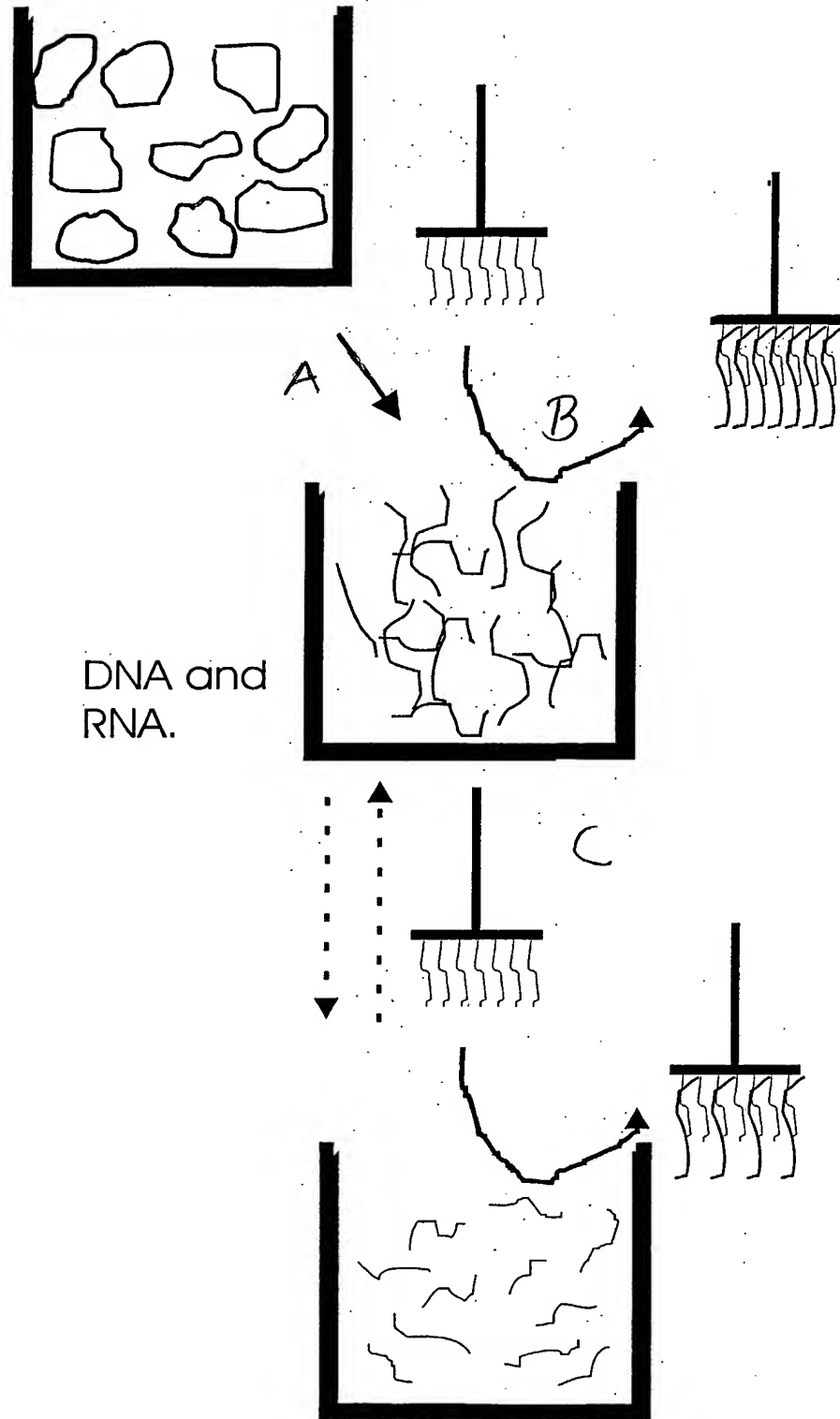
8/32

Fig. 8



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Fig. 9



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Fig. 10

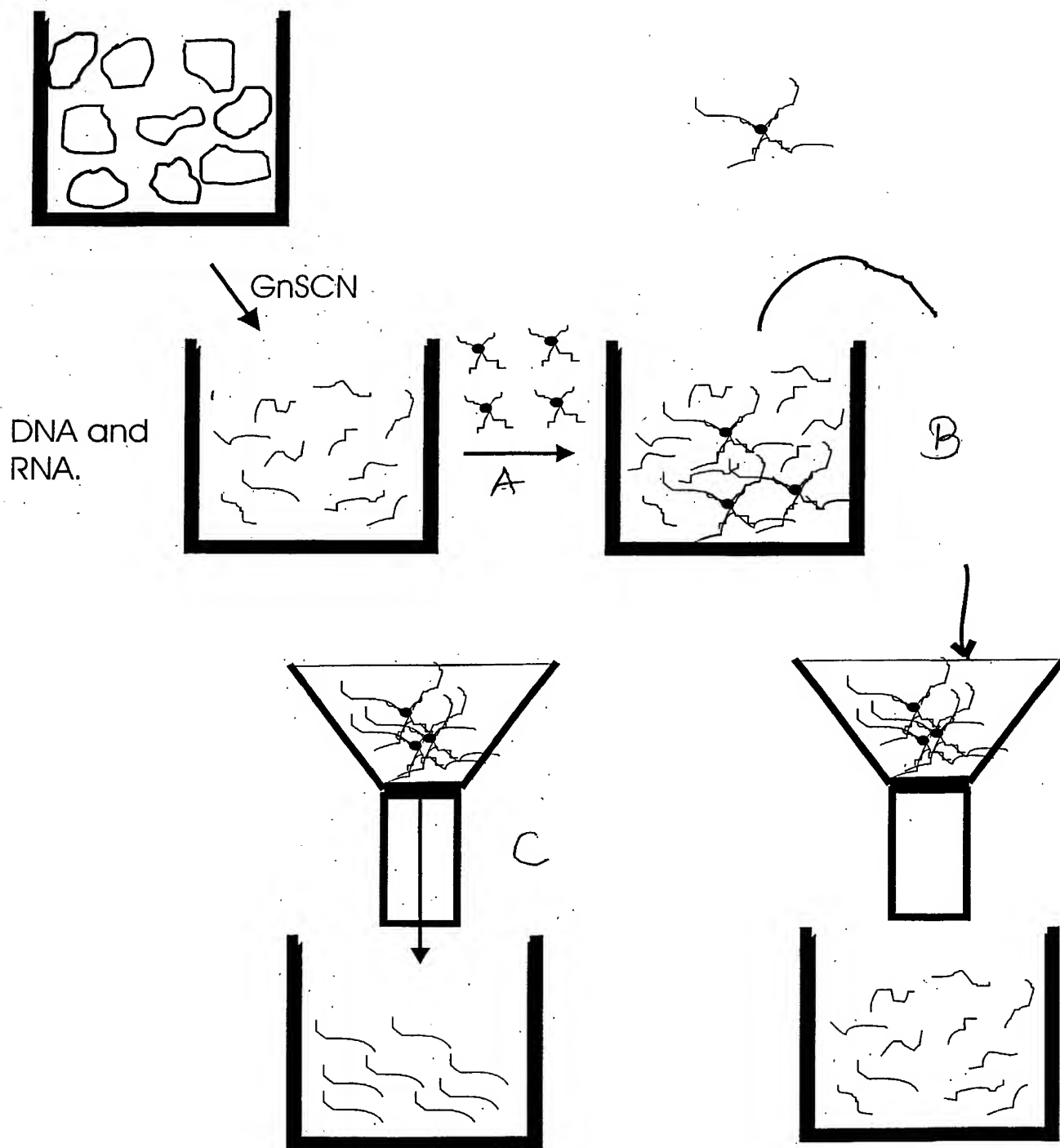


Fig. 11

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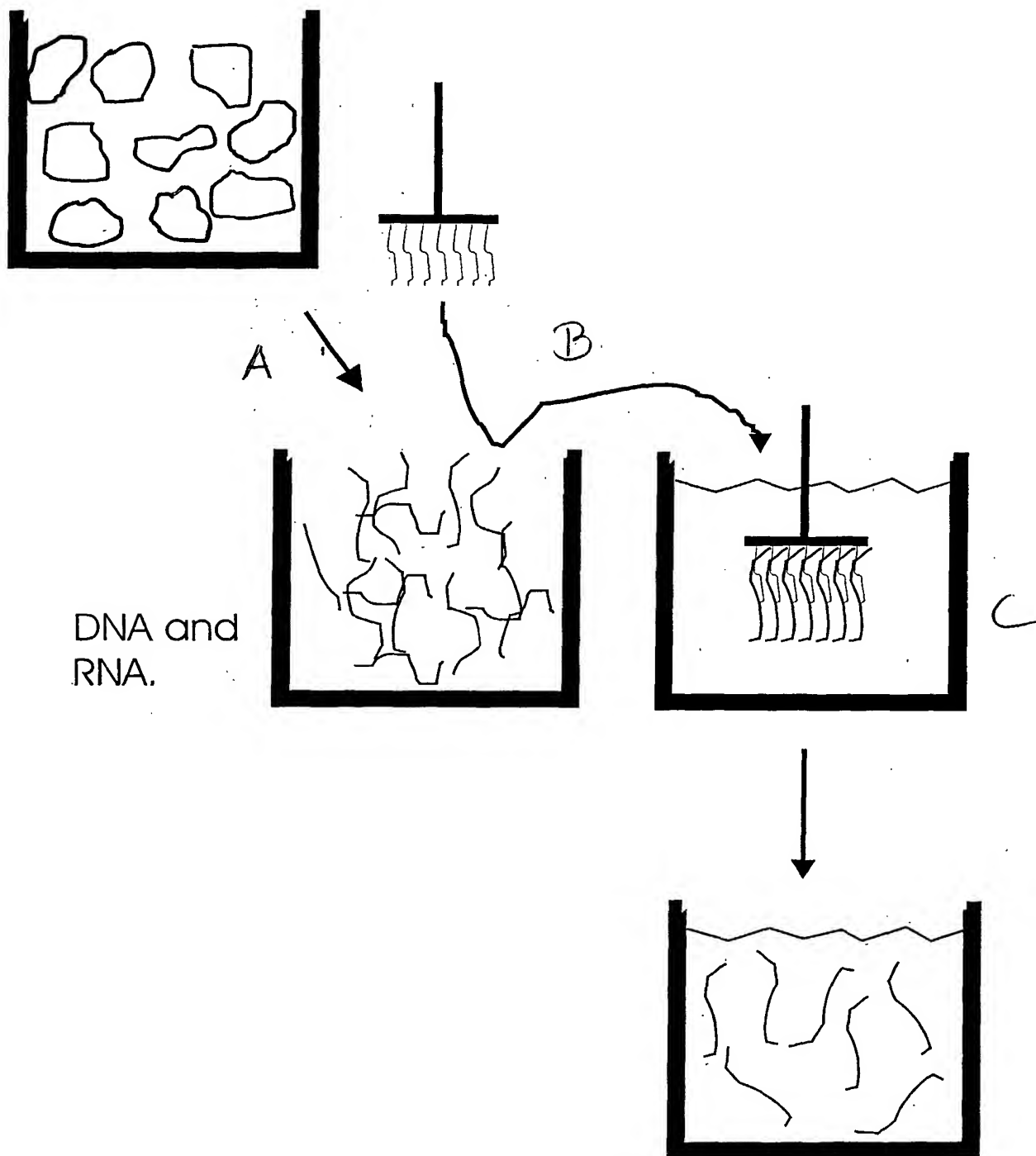
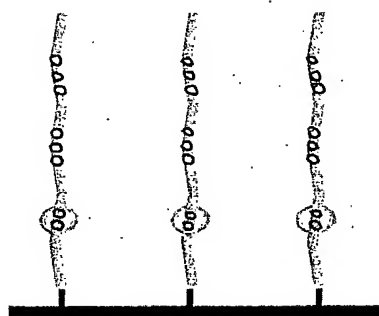
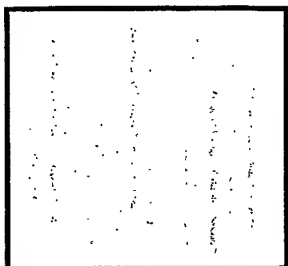


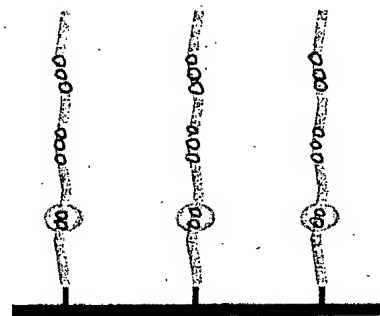
Fig. 12

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1

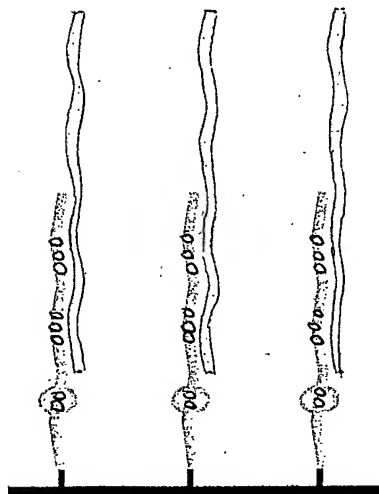


Spot 1

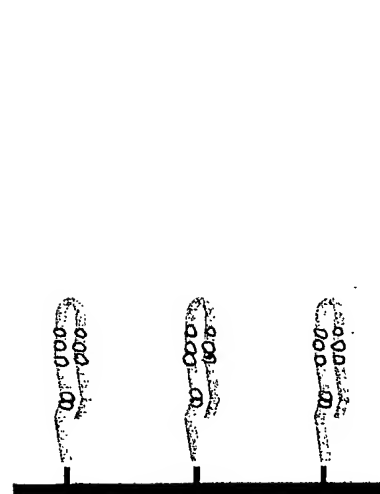


Spot 2

2



Spot 1



Spot 2



Fig. 13

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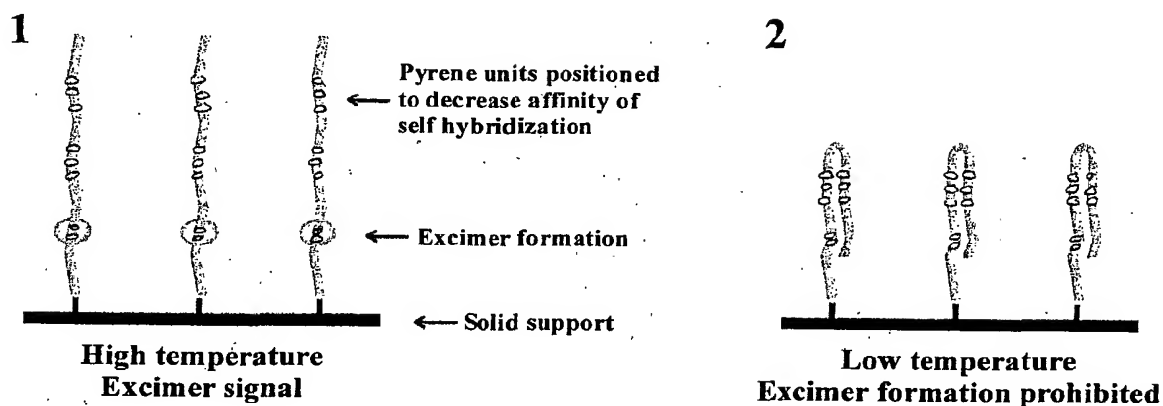
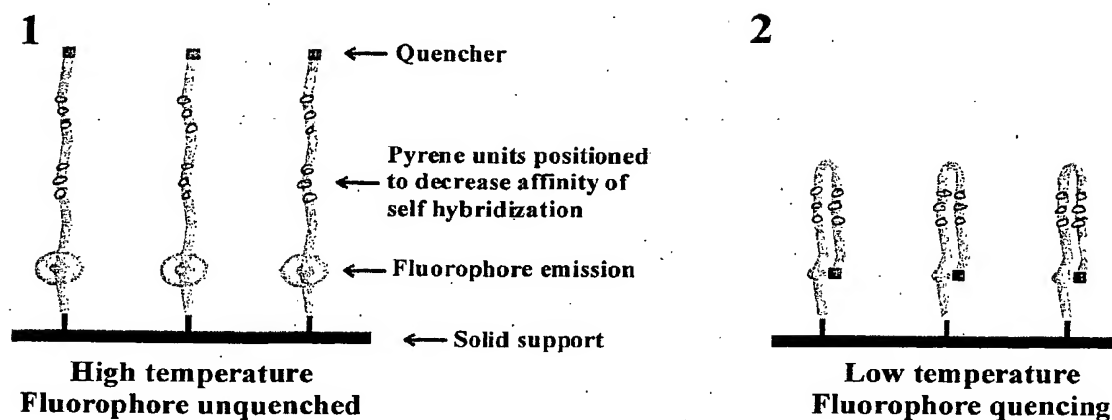
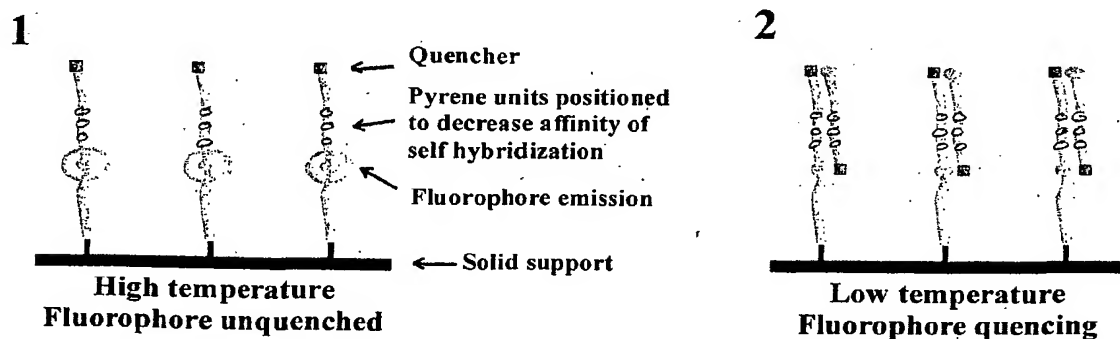
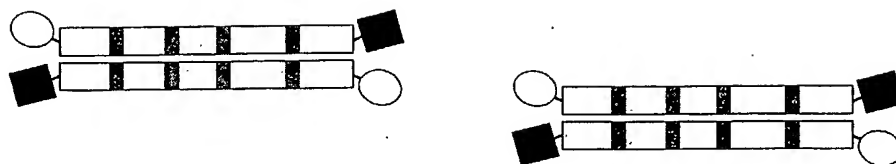
**Basal Chip states****A Excimer Chip****B Traditional Fluorophore Chip****C Beacon Sandwich Fluorophore Chip**

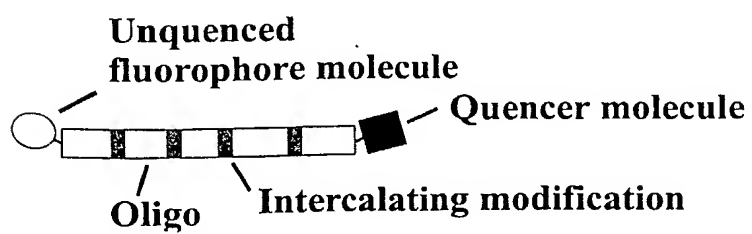
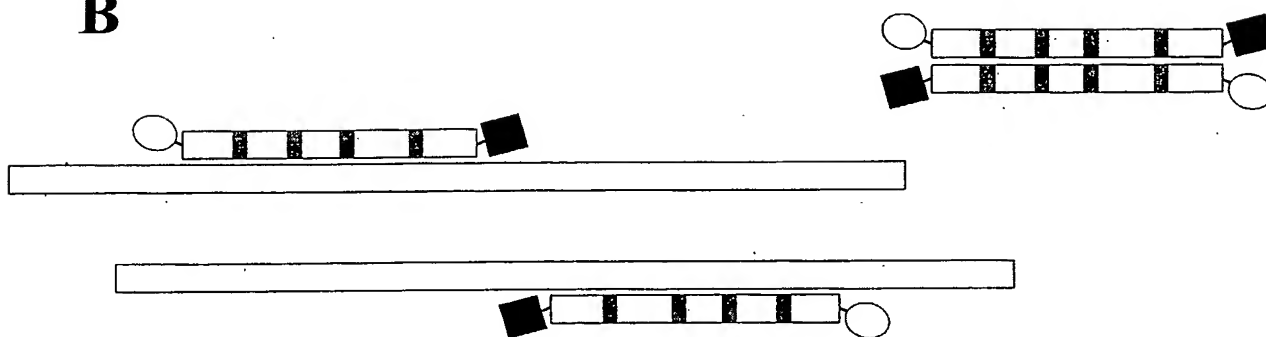
Fig. 14

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A



B



15/32

Fig. 15

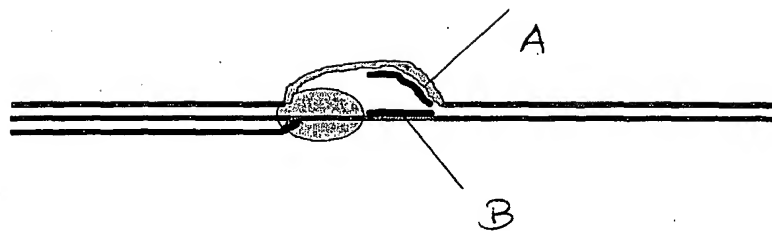


Fig. 16

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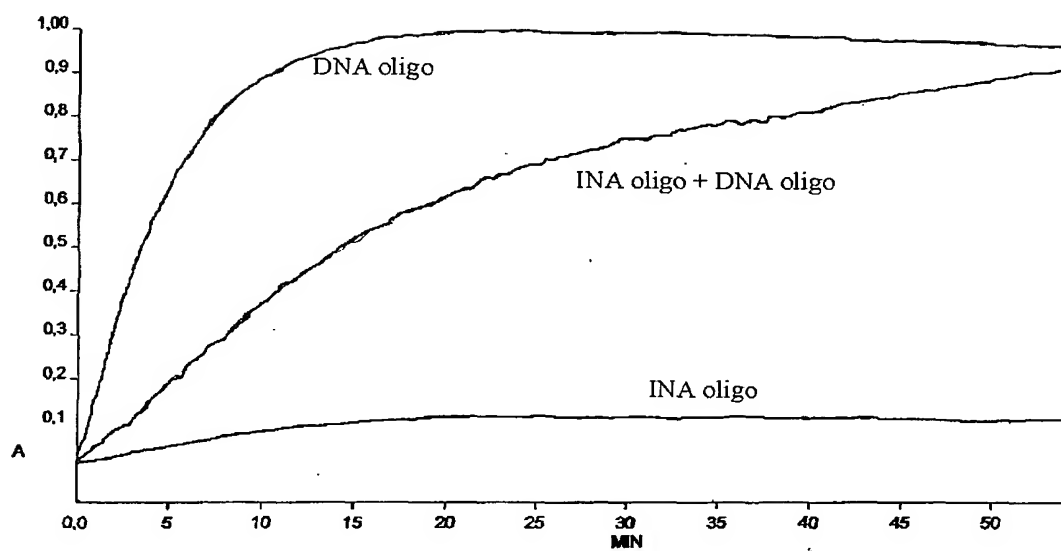
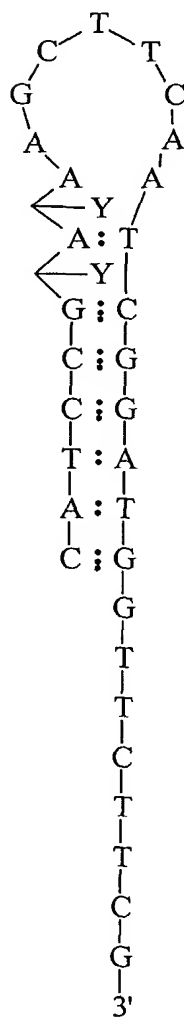


Fig. 17



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Fig. 18

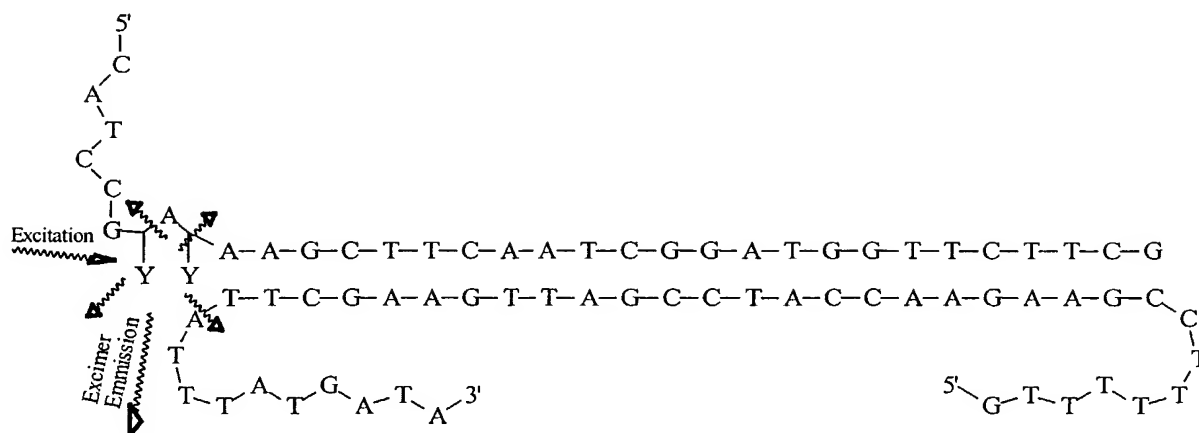
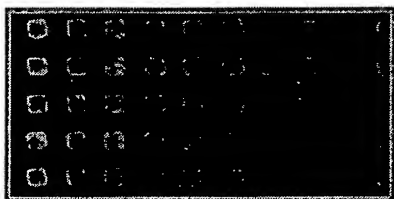
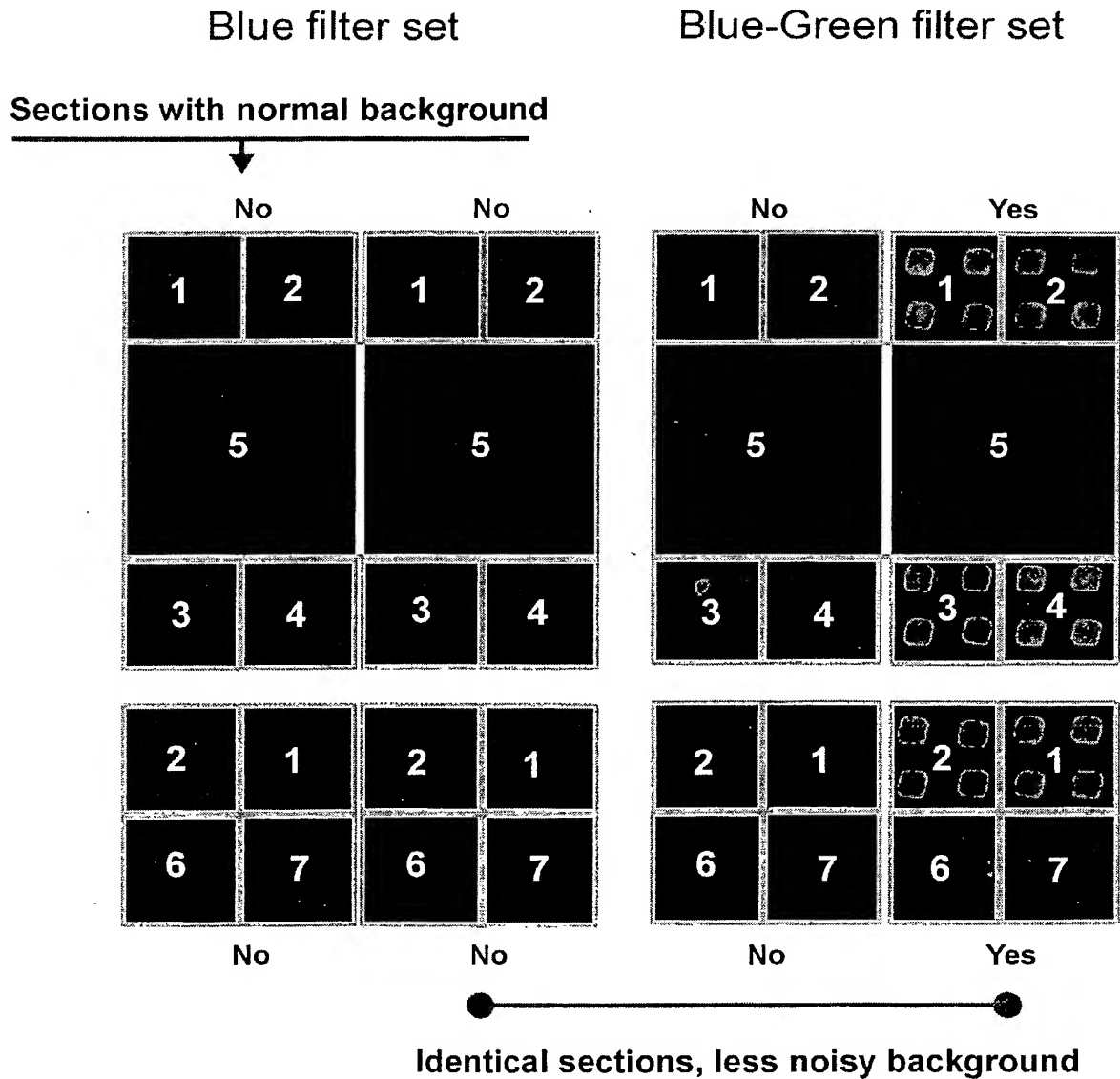


Fig. 19



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**Fig. 20** Testing oligo binding on Asper SAL slides



## Labels

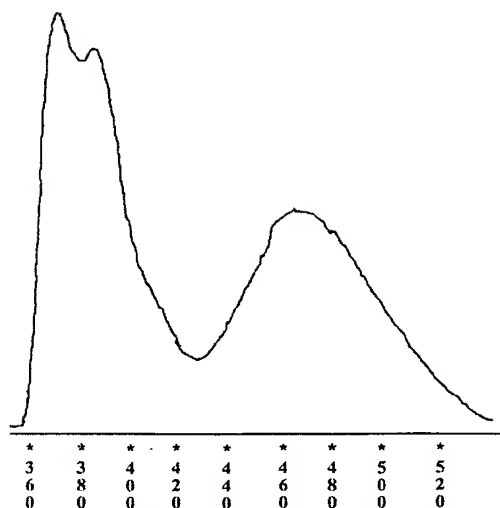
INA-oligos with linker & signal ( 1, 2, 3 & 4)

Short INA oligos, no linker or signal (5)

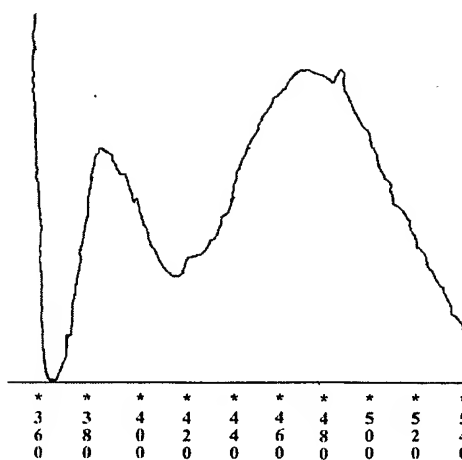
Buffer only (6), normal oligo with linker (7)

SYBR Green (Yes/ No)

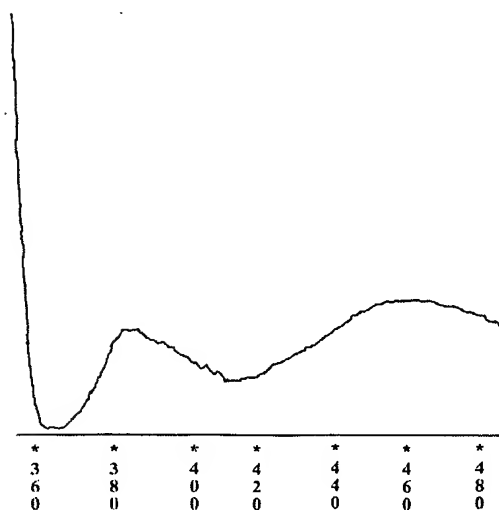
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**Fig. 21**

Exciplex between molecules X and Y  
when placed as next-nearest  
neighbors (sequence I)

**Fig. 22**

Exciplex fluorescence between the  
to molecules X and Y when placed  
as neighbours (sequence II)

**Fig. 23**

Exciplex fluorescence between  
molecules Y and Z when placed as  
neighbours

Fig. 24

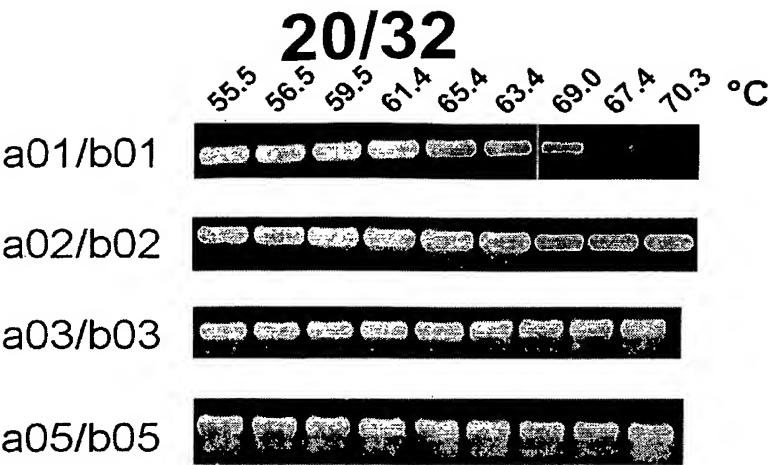


Fig. 25

Target 5'- GGGCTTTAAAGCCTCGCGGGGCTGACAGGTGAAATCGGGCGCGAAAGCTGTCGGGGTAGCGTCTGCACGCCCTAGGGGAT

Target 3'- CCCGAAATTTCGGAGCGCCCGGACTGTCCACTTTAGCGCGCCTTCGACAGCCCCATCGCAGAGCTGCGGGATGCCCTA

IO 1-1: 3'- ACTYTTAGYCCGYCGCCTTCGYA

IO 1-2: 3'- ACTYTTAGYCCGYCGCCTTCYGA

IO 1-3: 3'- ACTYTTAGYCCGYCGCCTTYCGA

IO 1: 5'- TGAATCGGCGCGGAAGCTYG

IO 3: 5'- TGAATCGGCGCGGAYAGCTYG

IO 5: 5'- TGAYAATCYGGCYGCGAYAGCTYG

Fig. 26

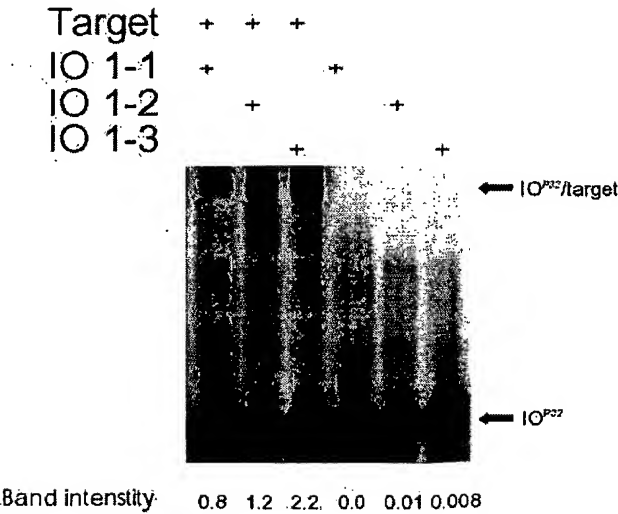






Fig. 29

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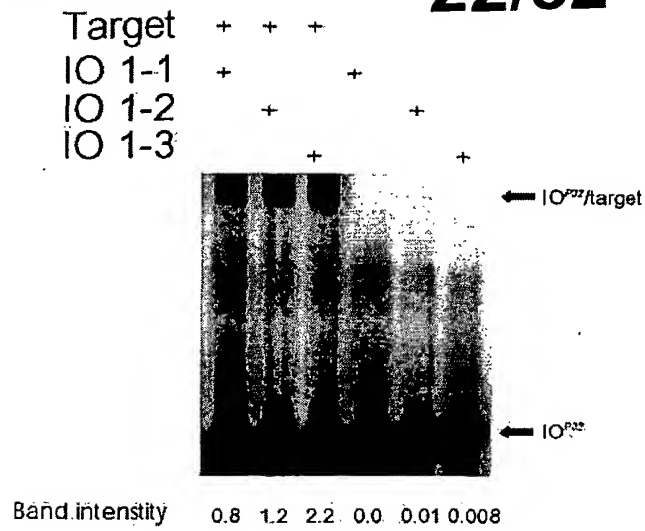
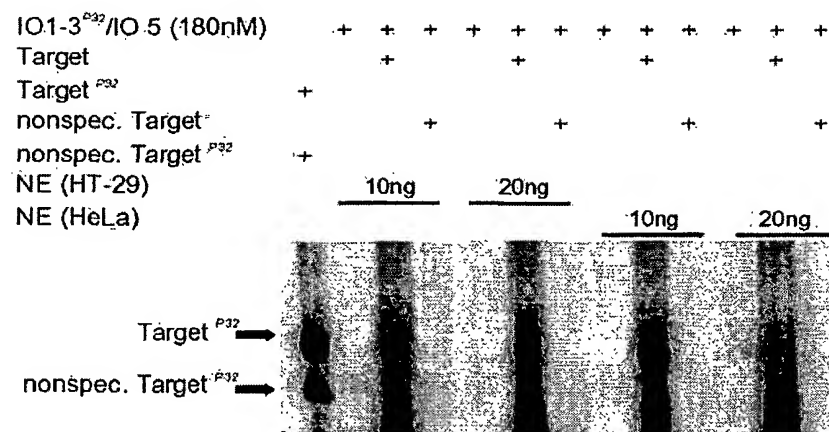
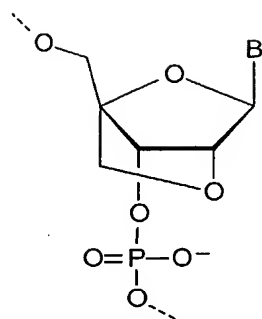


Fig. 30

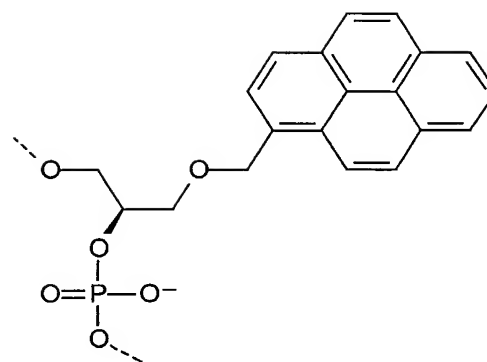


**Fig. 31**

Target <sup>P32</sup>	+	
Target	+	+
IO 1-3 <sup>P32</sup>	+	+
IO 1-3 <sup>P32</sup> /IO 5		+

**23/32****Fig. 32**

LNA monomer



INA monomer P

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Fig. 33

Table 1.

## Targets

I

5'-TGT GAT ATG CT

II

5'-TGT GAT<sup>L</sup> ATG CT

III

5'-TGT<sup>L</sup> GAT<sup>L</sup> AT<sup>L</sup>G CT

Entry	Probes	Thermal meltings with target I-III (°C)					
		I		II		III	
		$T_m$	$\Delta T_m$	$T_m$	$\Delta T_m$	$T_m$	$\Delta T_m$
1	3'-ACA CTA TAC GA	42.4	—	47.4	—	56.7	—
2	3'-ACA CTPA TAC GA	47.6	5.2	44.8	-2.6	52.9	-3.8
3	3'-ACA CTAP TAC GA	52.5	10.1	46.5	-0.9	54.5	-2.2
4	3'-ACA CPTA TAC GA	44.4	2.0	47.8	0.4	57.8	1.1
5	3'-ACA CTA TPAC GA	48.8	6.4	52.0	4.6	55.2	-1.5
6	3'-ACAP CTA TAC GA	50.6	8.2	54.2	6.8	55.1	-1.6
7	3'-ACA CTA TAPC GA	50.3	7.9	54.8	7.4	53.1	-3.6
8	3'-ACPA CTA TAC GA	45.0	2.6	50.3	2.9	53.7	-3.0
9	3'-ACA CTA TAC PGA	44.4	2.0	49.2	1.8	57.2	0.5
10	3'-APCA CTA TAC GA	47.5	5.1	52.0	4.8	63.5	6.8
11	3'-ACA CTA TAC GPA	49.6	7.2	54.3	6.9	62.7	6.0
12	3'-ACA CTA TAC GAP	48.7	6.3	53.8	6.4	60.4	3.7
13	3'-ACAP CTA TPAC GA	54.8	12.4	60.0	12.6	52.9	-3.8

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**Fig. 34**

**Table 2.**

**Probes**

T<sub>4</sub>-DNA 5'-GATAA TTTT TTATC  
 T<sub>4</sub>-LNA 5'-GAT<sup>L</sup>AA TTTT T<sup>L</sup>T<sup>L</sup>AT<sup>L</sup>MeC<sup>L</sup>  
 P<sup>2</sup>-LNA 5'-GPAT<sup>L</sup>AA TTTT T<sup>L</sup>T<sup>L</sup>AT<sup>L</sup>MeC<sup>L</sup>  
 P<sup>5</sup>-LNA 5'-GAT<sup>L</sup>APA TTTT T<sup>L</sup>T<sup>L</sup>AT<sup>L</sup>MeC<sup>L</sup>  
 P-P-LNA 5'-GPAT<sup>L</sup>APA TTTT T<sup>L</sup>T<sup>L</sup>AT<sup>L</sup>MeC<sup>L</sup>

**Targets**

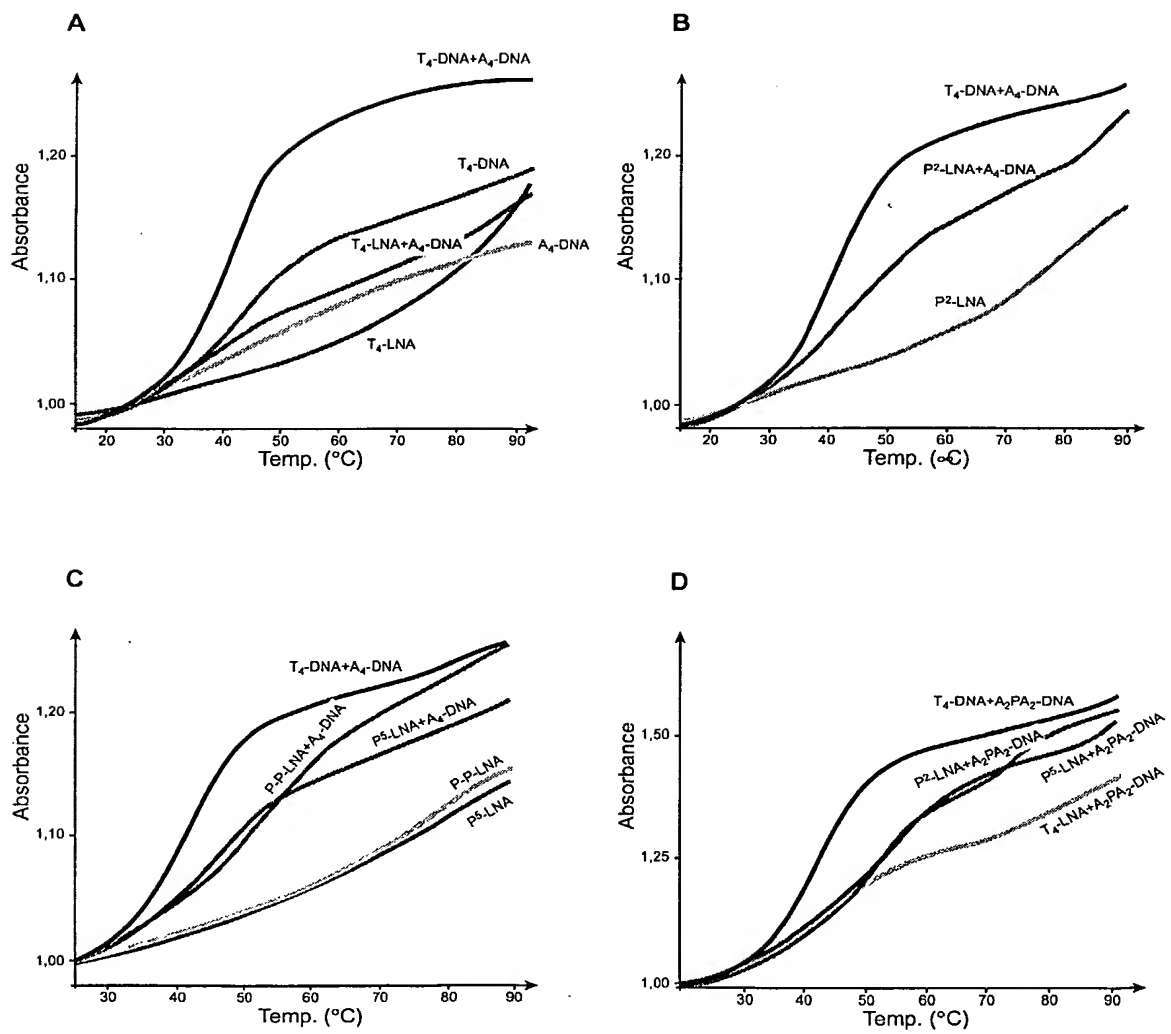
A<sub>4</sub>-DNA 3'-CTATT AAAA AATAG  
 A<sub>2</sub>PA<sub>2</sub>-DNA 3'-CTATT AAPAA AATAG  
 (APA)<sub>2</sub>-DNA 3'-CTATT APAAPA AATAG

Probes	Targets			
	Without target	A <sub>4</sub> -DNA	A <sub>2</sub> PA <sub>2</sub> -DNA	(APA) <sub>2</sub> -DNA
Without probe	—	<20	<20	<20
T <sub>4</sub> -DNA	37.2	37.2	40.3	NT
T <sub>4</sub> -LNA	>80	37.3	44.7	42.0
P <sup>2</sup> -LNA	(81.1)	46.4	49.9	NT
P <sup>5</sup> -LNA	(71.4)	43.3	51.9	NT
P-P-LNA	(69.1)	53.6	61.8	83.3

ND, not determined.

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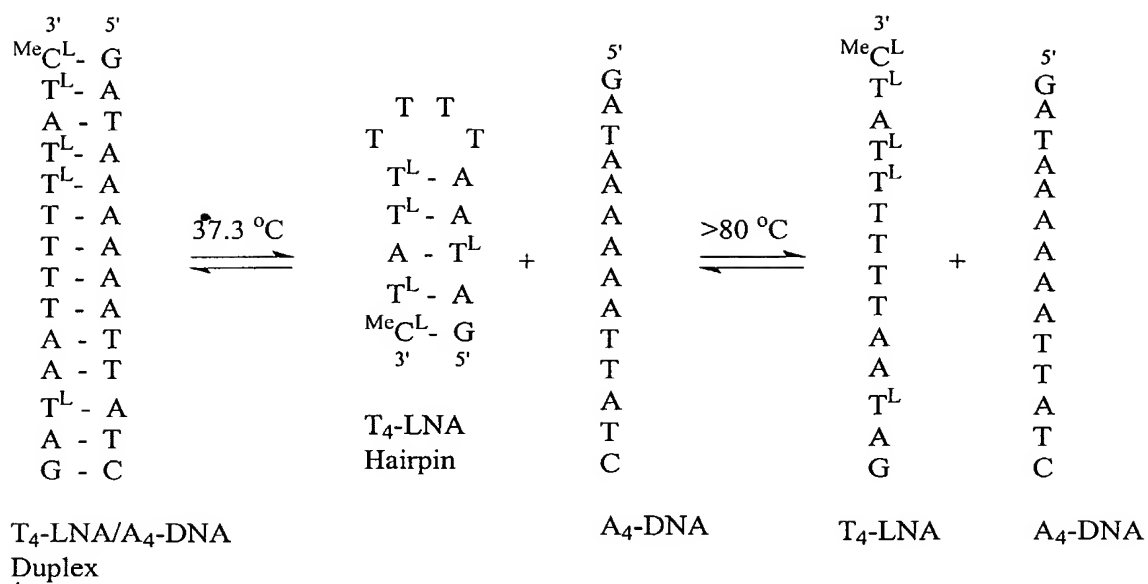
Fig. 35



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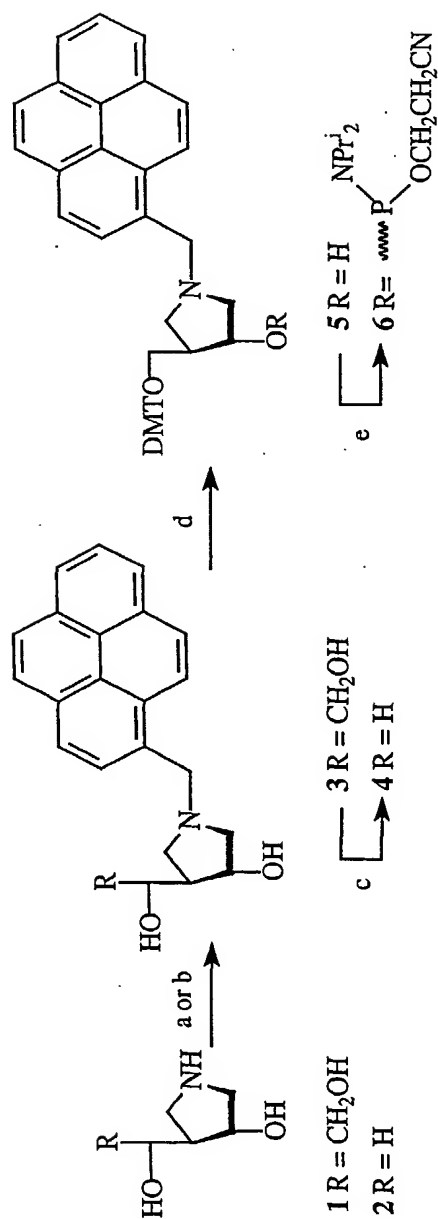
Fig. 36

Scheme 1



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Fig. 37



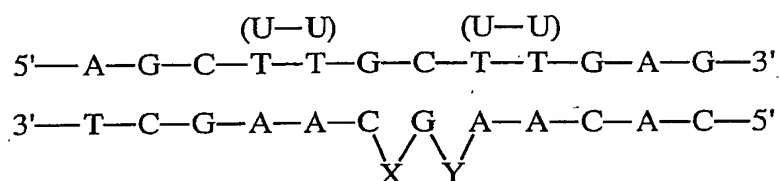
**Scheme 1** (a) 1-(chloromethyl)pyrene,  $Et_3N$ , DMF; (b) 1-pyrenecarbaldehyde,  $NaCNBH_3$ , DMF/EtOH (3:1); (c) 1)  $NaIO_4$ , 2)  $NaBH_4$ ; (d) DMTCI, pyridine; (e)  $NC(CH_2)_2OP(NPr)_2$ ,  $N,N$ -diisopropylammonium tetrazolide,  $CH_2Cl_2$ .



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Fig. 38

Sequences and Hybridization Data of Synthesised ODNs in DNA/DNA(RNA) Duplexes

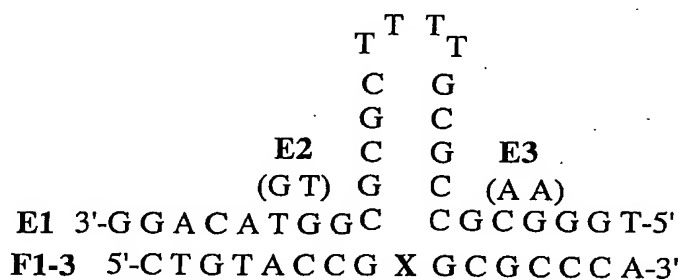


No	X	Y	$T_m$ (°C) (DNA/DNA)	$\Delta T_m$ (°C)	$T_m$ (°C) (DNA/RNA)	$\Delta T_m$ (°C)	$\Delta \Delta T_m$ DNA/RNA
A	—	—	43.0		42.2		
B	G	—	32.2	-10.8	32.6	-9.6	1.2
C	I	—	41.8	-1.2	32.2	-10.0	8.8
D	I	I	39.4	-1.2	21.8	-10.2	9.0

I = inserted nucleoside analogue 4,  $\Delta T_m$  = decrease in  $T_m$  per modification,  $\Delta \Delta T_m$  DNA/RNA = discrimination in  $T_m$  between DNA/DNA and DNA/RNA duplexes per modification

Fig. 39

Hybridization Data ( $T_m$  °C) for the DNA Three-Way Junction

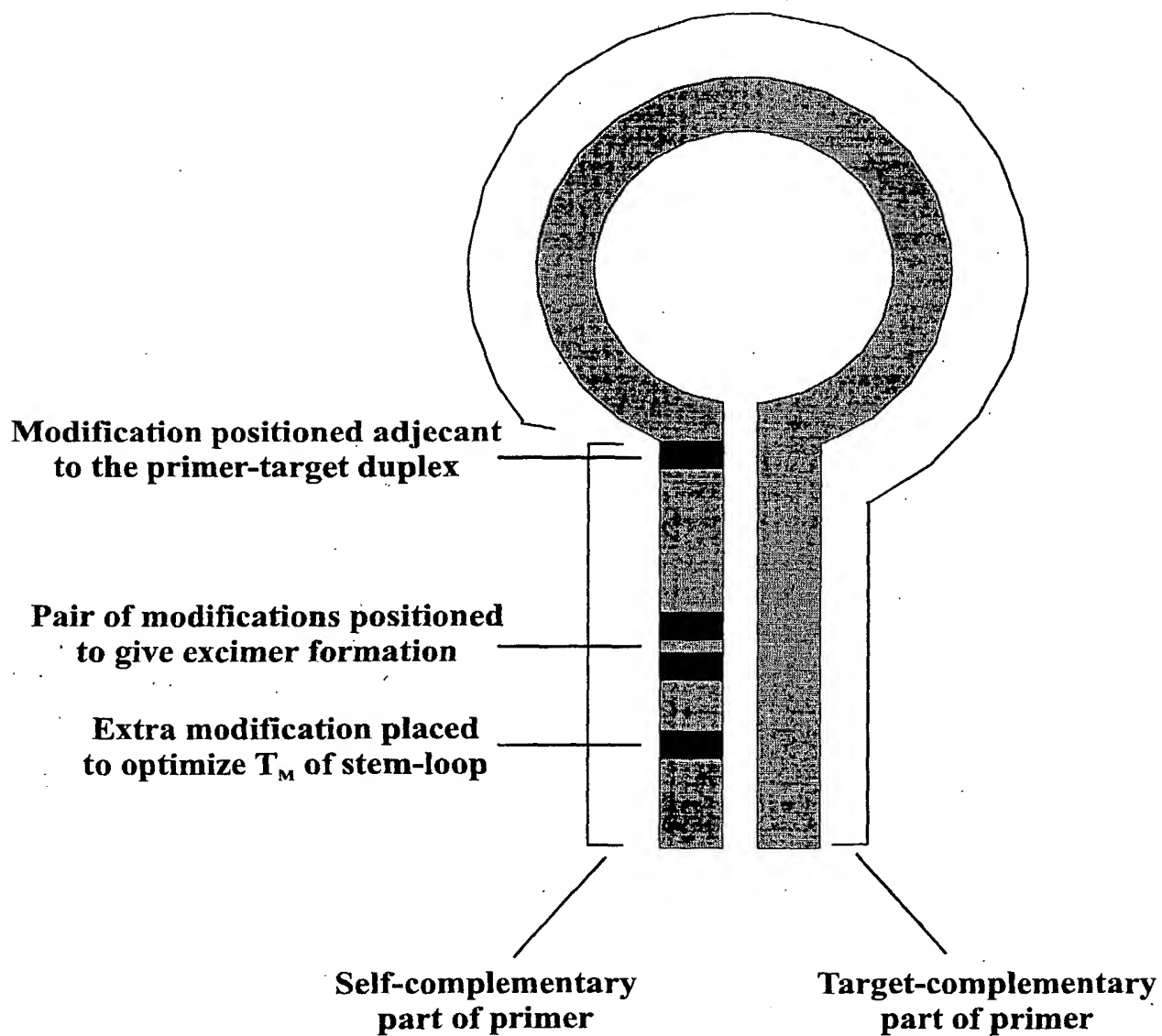


	F1 (X = 0)	F2 (X = A)	F3 (X = I)
E1	38.6	39.4	48.6
E2	<18.0	20.2	24.2
E3	<18.0	19.4	<18.0

I = inserted nucleoside analogue 4

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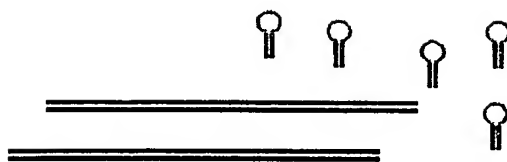
Fig. 40



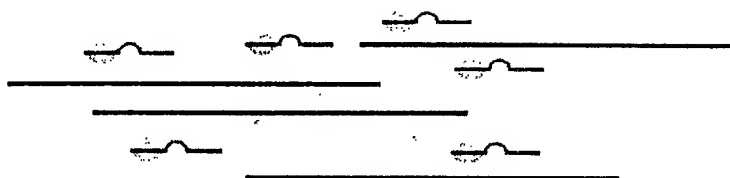
31/32

Fig. 41

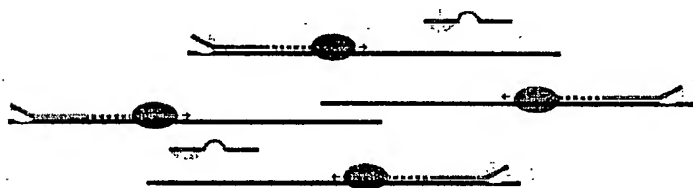
A



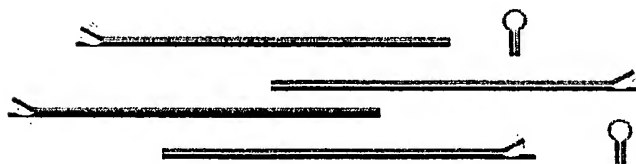
B



C



D



Beacon-design primer



Target-complementary part of oligo



Fluorescent oligo modifications



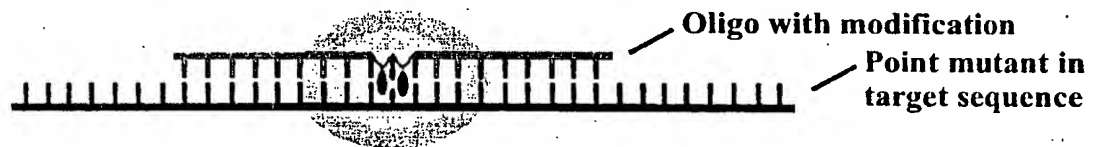
Target DNA



Amplified DNA

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Fig. 42

**A Complete complementarity****B Mismatch and excimer-formation**

- Y Fluorescent modification
- ↑ Point mutation mismatch
- ↑ wt base pairing